Vulval intraepithelial neoplasia

Cellular markers of progression and novel immunotherapy

Doctor of Medicine (M.D.)

University of London

2005

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Squamous intraepithelial neoplasia can develop at several sites in the lower genital tract and is associated with a risk of malignant progression. Women with vulval intraepithelial neoplasia (VIN) usually undergo treatment, not only to reduce the risk of subsequent carcinoma but also in an attempt to alleviate symptoms. Current therapies are often unpleasant and frequently ineffective, with many patients requiring multiple treatments for recurrent, recalcitrant disease. The central role of human papillomaviruses (HPVs) in the pathogenesis of cervical squamous neoplasia is now accepted. However, the potential mechanisms underlying disease progression in VIN are poorly understood making it difficult to define a high-risk group of patients in whom treatment would be most urgent.

In the work presented in this thesis, viral and genetic events of potential importance in the pathogenesis of vulval neoplasia have been identified. Using polymerase chain reaction (PCR) oncogenic HPVs were found within pre-invasive VIN lesions often, but not exclusively, as viral integrants. It would appear that viral integration might therefore be an early event in vulval oncogenesis. RNA *in situ* hybridisation has shown that the HPV is transcriptionally active and could therefore contribute to the subversion of normal cellular growth control mechanisms. Comparative genomic hybridisation (CGH) has identified chromosomal alterations occurring in a non-random fashion within VIN lesions. Several of these aberrations show striking similarities to those seen in invasive disease of both the vulva and cervix and may help to define a high-risk group of patients. Finally, the use of a novel viral vector vaccine to induce cell-mediated immune responses to non-structural HPV proteins in women with VIN is described. Vaccination can be associated with disease regression suggesting that development of effective immunostimulation could become an additional therapy for women with VIN.

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Abbreviations

A	Adenosine
AGIN	Anogenital intraepithelial neoplasia
AI	Allelic imbalance
APC	Antigen presenting cell (s)
APOT	Amplification of papillomavirus transcripts
bp	base pairs
BSA	Bovine serum albumin
C	Cytosine
CDK	Cyclin dependent kinase(s)
CDKI	Cyclin dependent kinase inhibitor(s)
CFS	Common fragile sites
CGH	Comparative genomic hybridisation
CIN	Cervical intraepithelial neoplasia
CIP	Calf intestinal alkaline phosphatase
CKI	Cyclin dependent kinase inhibitor
CMI	Cell mediated immunity/immune response
CNI	Copy number imbalance(s)
CTL	cytotoxic T-lymphocyte
CUSA	Cavitronic surgical aspiration
DAPI	4,6-Diamidino-2-phenylindole
dNTP	Deoxyribonucleotide triphosphate(s)
DOP	Degenerate oligonucleotide-primed
DTT	Dithiothreitol
dUTP	Deoxyuracil triphosphate
DNA	Deoxyribonucleic acid
FIGO	International Federation of Gynecology and Obstetrics
FISH	Fluorescence in situ hybridisation
G	Guanine
GTAC	Gene Therapy Advisory Committee
H/E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPV	Human papillomavirus
HR-HPV	High-risk human papillomaviruses
HRP	Horseradish Peroxidase
HSIL	High-grade squamous intraepithelial lesions
HSE	National Health and Safety Executive
ISH	In situ hybridisation
ISSVD	International Society for the Study of Vulvovaginal Disease

IFN	Interferon
IL	Interleukin
kb	Kilobase
LOH	Loss of heterozygosity
LBC	Liquid-based cytology
LCR	Long control region
LR-HPV	Low-risk HPV human papillomaviruses
LSIL	Low-grade squamous intraepithelial lesions
Mb	Megabase pairs
MCA	Medicines Control Agency
MHC	Major histocompatibility complex
MS	Microsatellite
MI	Microsatellite instability
NCR	Non-coding region
NK	Natural killer cell(s)
NNED	Non-neoplastic epithelial disorders
ORF	Open reading frame
Ori	Viral origin of replication
PDT	Photodynamic therapy
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	plaque forming units
pRb	Retinoblastoma protein
RE	Restriction enzyme
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
SSC	Salt Sodium Citrate
SCC	Squamous cell carcinoma
SF-PCR	Short-fragment PCR
T	Thymidine
Th	T-helper cell(s)
TLR	Toll-like receptor
TSA	Tyramide signal amplification
TSG	Tumour suppressor gene
U	Uracil
UV	Ultra-violet
URR	Upstream regulatory region
VIN	Vulval intraepithelial neoplasia
VLP	Virus-like particle

To my wife Emma, without whose love and support this thesis would never have been completed.

I wish to thank my supervisor Dr Jane Sterling, for her support and guidance since the inception of this work. I enjoyed my time working in the Division of Virology, University of Cambridge. Many members of the Division assisted or encouraged me in the completion of this thesis and I remain grateful to you all. Particular thanks must be given to Miss Amy Nicholson for her technical assistance and companionship. In addition, Dr Nicholas Coleman's group at the MRC Cancer Cell unit provided invaluable help in setting up the difficult technique of CGH.

Chapter 1 Vulval intraepithelial neoplasia

Intraepithelial neoplasia can develop in several anatomical sites in the lower genital tract and carries with it the risk of progression to malignancy. Squamous intraepithelial neoplasia of the vulva is difficult to treat and often causes troublesome symptoms for the patient. There is a need both to try to understand the pathophysiology of this disease as well as to develop new treatment modalities, thereby providing patients with the possibility of individualised management with treatments that are both more acceptable and effective.

1.1 Clinical aspects

1.1.1 Classification of intraepithelial disease of the Vulva

The first description of intraepithelial neoplasia of the vulval skin appears to have been in the early 1920's (Hudelo *et al.*, 1922). Since this time the condition has been described with a somewhat bewildering variety of terms, some of which are listed below.

- Bowen's disease of the vulva
- Bowenoid atypia & dysplasia
- Bowenoid papulosis
- Erythroplasia of Querat
- Carcinoma in situ of the vulva
- Hyperplastic dystrophy with severe atypia

In an effort to reduce clinical confusion, the Terminology Committee of the International Society for the Study of Vulvar Disease (ISSVD) recommended a standardised classification for intraepithelial neoplasia of the vulva (Wilkinson *et al.*, 1986). This classification is detailed in (Table 1-1). This thesis will be confined to intraepithelial neoplasia of the squamous type known as vulval intraepithelial neoplasia.

The classification for squamous intraepithelial neoplasia of the vulva is analogous in terminology to the 'three-tier' (cervical intraepithelial neoplasia or

CIN) system employed by some countries for disease of the uterine cervix (Richart, 1973). Some investigators have extrapolated this similarity, referring to VIN 2/3 as 'high-grade' disease in a similar fashion to the 'two tier' Bethesda classification (Solomon *et al.*, 2002) for cervical disease. In contrast to disease in the cervix, the majority of preinvasive lesions diagnosed are high-grade lesions, usually VIN 3. Although progression from VIN 1 to VIN 3/SCC has been documented (Barbero *et al.*, 1990), clear evidence for a stepwise progression is lacking and the relevance of a diagnosis of VIN 1 therefore remains controversial. This may reflect the heterogeneous nature of disease that falls within this classification. Difficulties in the histological grading of lesions will be discussed in detail (section 1.1.1.1)

a. Squamous		*b. Non-squamous*
VIN 1		Paget's disease
VIN 2	} High-grade	Melanoma *in situ*
VIN 3		

Table 1-1 – 1986 ISSVD classification of intraepithelial neoplasia of the vulva with additional annotation showing so-called 'high-grade' disease

1.1.1.1 Histological subtypes of VIN

In addition to the histological grading system described above, VIN may be subdivided on a morphological basis into similar categories to vulval SCC. The majority of cases show classic features of lower genital tract intraepithelial neoplasia, with immature cells showing a high nuclear to cytoplasmic ratio, hyperchromatic nuclei and occasional mitotic figures extending most of the way through the epithelium. The strong association of this type of VIN with human papillomavirus (HPV) infection will be discussed later (section 1.1.4.1). This group has been further divided into 'basaloid' (undifferentiated) and 'warty' (Bowenoid) sub-types (Park *et al.*, 1991a) dependent on the degree of differentiation, koilocytosis and surface contour. There would appear to be considerable overlap between these two sub-types with many lesions showing a 'mixed' histological picture (Hørding *et al.*, 1995).

a) b)

c)

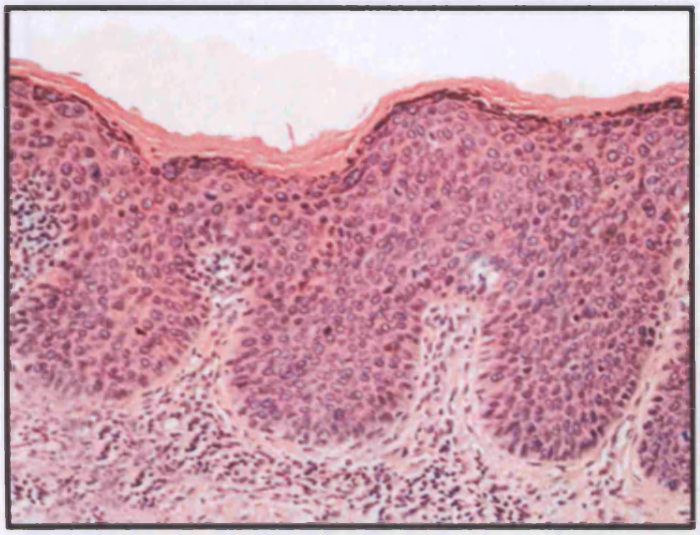


Figure 1-1. 'Classical' or 'undifferentiated' vulval intraepithelial neoplasia

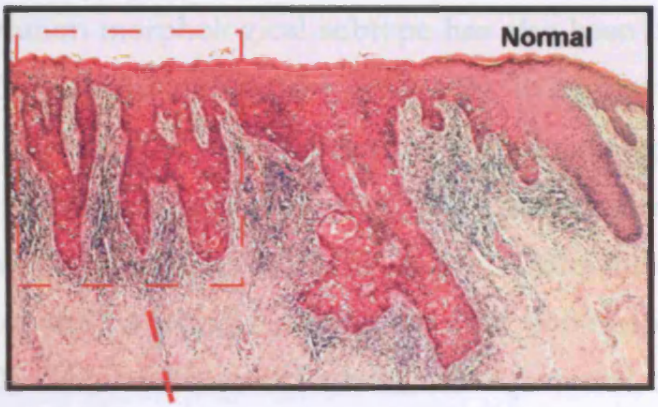
a) H/E section seen at low power showing normal stratified squamous epithelium on the left adjacent to full thickness VIN (VIN 3) on the right of the image

b) Adjacent section stained with the proliferation marker MIB1. In the normal vulval skin proliferation is confined to the basal layer of the epidermis. In contrast, within the areas of VIN 3, proliferation is seen throughout the full thickness of skin.

c) High-grade VIN (VIN 3) - immature cells showing a high nuclear:cytoplasmic ratio, hyperchromatic nuclei and occasional mitotic figures extending the whole way through the epidermis. This type of VIN shows a strong association with human papillomavirus infection, notably by the oncogenic type 16.

(Images a) and b) courtesy of Dr R. Moseley, Addenbrooke's Hospital, Cambridge)

a)



b)

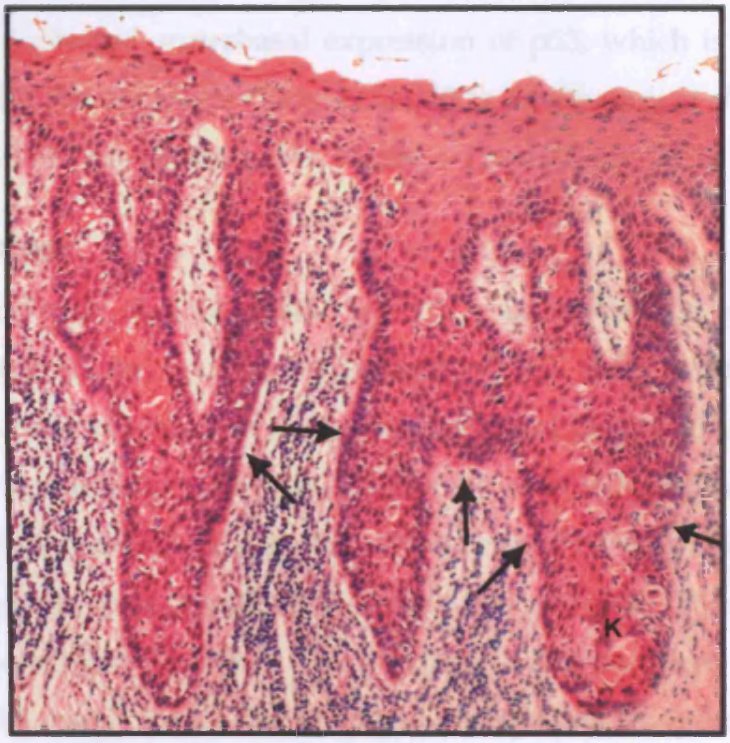


Figure 1-2. 'Differentiated' VIN

a)Low power view of H/E section. Normal vulval skin evident on the right hand side of the image. Left hand sides shows 'differentiated' VIN. The diagnosis of 'differentiated' VIN may be difficult.

b) Area indicated by box at higher magnification to show features of 'differentiated' VIN. Preservation of epithelial maturation in upper layers with atypia limited to basal cell layers (indicated by arrows). Abnormal keratinisation seen in lower layers of epidermis (K). 'Differentiated' VIN accounts for <5% prospectively identified VIN and is commonly associated with non-neoplastic epithelial disorders or keratinising SCC. Unlike 'classical' VIN, 'differentiated' VIN is commonly HPV-negative.

A second, less common morphological subtype has also been described and is referred to as 'differentiated' or 'simplex-type' VIN. In contrast the 'classic' or 'undifferentiated' type of VIN, these lesions show minimal atypia of the upper cell layers with proliferation accompanied by preservation of epithelial maturation and atypia limited to basal cell layers. Differentiated VIN rarely occurs in isolation and is usually associated with non-neoplastic epithelial disorders (NNED) such as lichen sclerosus and squamous cell hyperplasia (Leibowitch *et al.*, 1990; Rouzier *et al.*, 2001) or the keratinising form of vulval SCC. Differentiated VIN is difficult to diagnose and accounts for <5% of prospectively identified VIN (Haefner *et al.*, 1995). Cases tend to be negative for HPV and show increased suprabasal expression of p53, which is of potential use in establishing the diagnosis (Yang *et al.*, 2000; Al-Ghamdi *et al.*, 2002). The different types of VIN are illustrated in Figure 1-1 and Figure 1-2.

1.1.2 Epidemiology

VIN has an incidence of 2.1/100,000 woman years (Sturgeon *et al.*, 1992). Originally considered an uncommon disease of the older woman, several authors have recently reported an increasing incidence particularly in younger women. In their retrospective study of 113 women with VIN, Jones and Rowan found a fall in the mean age of affected women from 52.7 years for the period 1961-80 to 35.8 years for the period 1981-93 (Jones *et al.*, 1994). This finding has recently been confirmed by other investigators in the southern hemisphere (Thuis *et al.*, 2000), whilst data from European institutions have shown a three- to fourfold increase in the incidence of VIN (Herod *et al.*, 1996; Joura *et al.*, 2000). The result of an institutional retrospective study comparing the incidence of VIN 2/3 for two four year periods in consecutive decades (1985-88 and 1994-97) demonstrates the dramatic increase for the younger age group (Joura *et al.*, 2000 - illustrated in figure 1-3).

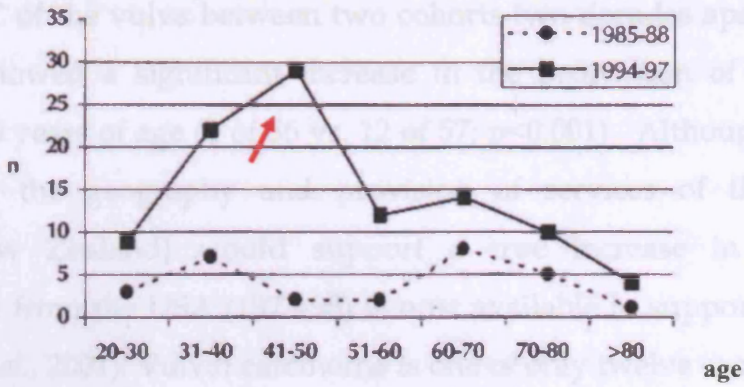


Figure 1-3. Incidence of high-grade VIN from two four year periods in consecutive decades. Arrow indicates dramatic increase in younger age group (Joura *et al.*, 2000)

Population data from Norway has also demonstrated an increase in the incidence of intra-epithelial neoplasia over the last 40 years (Iversen *et al.*, 1998). In contrast to the institutional studies already discussed, the authors found this increase to be spread across all age groups. They therefore suggest that the increase in incidence may simply represent increased awareness and subsequent diagnosis of VIN. Whilst institutional studies are particularly prone to observer and referral bias, such criticisms do not readily explain the preponderance of younger cases found in these studies. Furthermore, additional population data from the Surveillance, Epidemiology and End Results (SEER) program in the USA not only reveals a doubling in the rate of VIN but confirms that the largest (threefold) increase was seen for those women under the age of 35 years (Sturgeon *et al.*, 1992).

An increase in pre-invasive disease might be expected to be accompanied by a subsequent rise in the incidence of invasive squamous cell carcinoma (SCC) of the vulva. Until recently this effect had not been observed (Sturgeon *et al.*, 1992; Iversen *et al.*, 1998), lending further support to the view that the 'reported' rise in VIN was artefactual. Although latency could delay the appearance of such an effect, progression from VIN to carcinoma appears to be faster than for SCC of the cervix, usually occurring in less than eight years (Jones *et al.*, 1994; Herod *et al.*, 1996; Thuis *et al.*, 2000). A retrospective study (Jones *et al.*, 1997) comparing

the rates of SCC of the vulva between two cohorts two decades apart (1965-74 and 1990-94) showed a significant increase in the proportion of SCC cases younger than 50 years of age (1 of 56 vs. 12 of 57; $p<0.001$). Although this data is institutional, the geography and provision of services of the country concerned (New Zealand) would support a true increase in incidence. Population data from the USA (1973-98) is now available to support this early study (Howe *et al.*, 2001). Vulval carcinoma is one of only twelve cancers whose incidence rates have increased from 1992-98. In contrast to the usual older age group for this carcinoma, the increase was predominantly in women under 65 years of age. More recently still, a retrospective Canadian study confirmed a significant rise in vulval SCC affecting young women (<40years) over the study period 1975-1998 (Al-Ghamdi *et al.*, 2002).

1.1.3 VIN as a precursor of malignancy

The natural history of VIN is controversial. There seems little doubt that high-grade disease represents a true pre-invasive lesion but the quoted risk of progression varies greatly between studies (Table 1-2).

Study	*Progression (Number of cases/cohort)*	*%*	*Mean age*	*Interval /years*	*Comment*
(Buscema *et al.*, 1980)	2/102	2.0	79	n/a	immunosuppressed
(Crum *et al.*, 1984)	5/39*	12.8	55	<1-10*	median <1 year
(Hørding *et al.*, 1991)	3/19	15.8	n/a	3-5	
(Jones *et al.*, 1994)	11/113	9.7	53	2-18	median 5 years
(Herod *et al.*, 1996)	9/133	6.8	49	2-9	median 4.5yr
(Kuppers *et al.*, 1997)	4/59	6.8	n/a	n/a	
(Thuis *et al.*, 2000)	1/40	2.5	30	4	
(McNally *et al.*, 2002)	3/101	3.0	n/a	6-7	
Overall		**7.4**	**%**		

Table 1-2. Varying rates of progression for high-grade VIN. * It should be noted that progression within a year may imply poor initial treatment or a missed diagnosis and that 2/41 patients in this study were lost to follow-up.

What is not apparent from this data are that most studies involved populations of treated individuals and that progression in these studies therefore represents a risk of malignancy despite treatment. Data from untreated populations are scarce and unlikely to be acquired in the future. Jones *et al* provide an insight into the possible true natural history of disease (Jones *et al.*, 1994). Their study includes eight untreated individuals. When these patients are considered as a separate group, the risk of malignant progression appears rather more alarming at 87.5% (7/8 patients progressed to malignancy, six within 8 years). This compared to a figure of 3.8% in the treated group. Caution must be used when interpreting this difference as half the untreated group had previously been treated with pelvic radiotherapy and could be regarded as being at increased risk for lower genital tract malignancy. However, it remains likely that the true rate of progression of untreated VIN will be considerably in excess of the oft-quoted risk of 5-10%. Others have estimated the relative risk of developing anogenital carcinoma following a diagnosis of VIN to be as high as 29.8 (95%CI 7.3-263.4) (Sherman *et al.*, 1988).

The hypothesis that aggressive treatment of VIN may be reducing the apparent risk of vulval SCC is supported by studies of excisional biopsy specimens. Detailed histopathological assessment of specimens from lesions thought pre-operatively to be clinically intraepithelial (ie. VIN) has shown invasive carcinoma to be present in 16.4 to 22% of cases (Chafe *et al.*, 1988; Hørding *et al.*, 1995; Modesitt *et al.*, 1998; Husseinzadeh *et al.*, 1999; Thuis *et al.*, 2000). The majority of invasive lesions found represent superficial invasion (<1mm, FIGO stage 1A) and would be expected to have been cured by excisional treatment alone. Attempts to selectively demonstrate invasive areas using immunohistochemistry for Ki-67 (a proliferation marker) have so far been unsuccessful (Modesitt *et al.*, 2000).

The incidence of SCC of the vulva in younger women is increasing (section 1.1.2). In the cohort study already described (Jones *et al.*, 1997), women below the age of 50 years were significantly more likely to have VIN associated with their tumour than women over 50 years (10/13 vs. 13/100; $p<0.001$). Tumours

with adjacent VIN are usually 'basaloid' or 'warty' in type and frequently associated with HPV infection (Toki *et al.*, 1991; Hørding *et al.*, 1993; Kurman *et al.*, 1993; Al-Ghamdi *et al.*, 2002). When taken together, these findings support a role for undifferentiated VIN in the development of SCC of the vulva in the younger patient. Even stronger evidence of VIN as a precursor lesion is now available from molecular genetic studies. X-chromosome inactivation analysis (Tate *et al.*, 1997; Rosenthal *et al.*, 2002) and microsatellite markers (Rosenthal *et al.*, 2002) have been used to demonstrate the clonal nature of VIN and SCC associated with VIN in both contiguous and multifocal disease (section 1.2.3.2).

1.1.3.1 Spontaneous regression of high-grade disease

The recognition that 'severe atypia' could be reversible led to the use of separate descriptions such as 'Bowenoid papulosis' (Wade *et al.*, 1979) and 'Reversible vulval atypia' (Friedrich, 1972). Concern regarding the risk of malignant progression has led to aggressive intervention with many clinicians choosing to treat high-grade disease surgically (either by ablative or excisional methods). Limited data therefore exists regarding the rate of spontaneous regression of high-grade disease. In early studies of 'VIN' (Friedrich *et al.*, 1980; Bernstein *et al.*, 1983), spontaneous regression was surprisingly common (5/9 and 5/13 untreated individuals respectively) although investigators were still unable to predict those cases in whom regression was most likely (Bernstein *et al.*, 1983). The results must be interpreted with some caution given the inconsistent classifications used at the time, but in retrospect these individuals share many of the characteristics (Table 1-3) identified in a more recent review of 14 cases of VIN 2-3 in which spontaneous regression occurred (Jones *et al.*, 2000). Despite the lack of a histological endpoint in this study (regression was 'confirmed' by vulvoscopy alone), it is interesting to note the timescale for regression in these cases. The median time from diagnosis to complete disappearance of the lesions was 9.5 months.

Common features of high-grade disease associated with spontaneous regression
Young (*median age 19.5 –21 years)
Non-white
Associated with pregnancy/recent pregnancy
Atypical condyloma/recent condyloma
Multifocal
Papular
Hyperpigmented lesion

Table 1-3. Clinical features assosciated with disease regression.

(*median age from Friedrich *et al.*, 1980; Jones *et al.*, 2000)

Given the increasing incidence of VIN amongst younger women and the morbidity (physical and psychosexual) associated with surgical treatments, these features are especially relevant. It would seem appropriate to consider a period of 1-2 year's observation (by an experienced clinician) for such a patient. The discovery that cytological regression in CIN is preceded by clearance of HR-HPV infection types (Nobbenhuis *et al.*, 2001), would suggest that HPV testing (qualitative or quantitative) may be prognostically useful, at least for multifocal undifferentiated VIN.

1.1.4 Aetiology and pathogenesis of VIN

It is likely that 'VIN' may actually encompass at least two pathological entities (section 1.1.1.1). The vast majority of studies involve 'classical' or undifferentiated VIN. In this section, aetiological factors and aspects of disease pathogenesis will be considered further.

1.1.4.1 Human papillomavirus infection and VIN

In common with intra-epithelial neoplasia at other ano-genital sites, 'classical' VIN shows a strong association with HPV infection. The potential mechanisms of HPV oncogenesis will be discussed at a later point (section 1.2.1). Before considering the reported association of VIN and HPV, it is important to

understand that the reported incidence of HPV infection will vary with the method chosen to make this diagnosis.

1.1.4.1.1 The detection of HPV infection

In the simplest studies HPV infection was assumed from the finding of koilocytes on histological examination of lesional tissue. Histological assessment of VIN lesions suggests that 31-78% of lesions are associated with HPV infection (Herod *et al.*, 1996; I.S.G.V.D., 1996; McNally *et al.*, 2002).

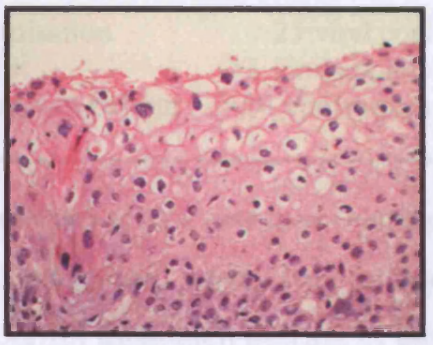


Figure 1-4. Koilocytes. Hyperchromatic, irregular nuclei and vacuolated cytoplasm giving the classical 'halo' type appearance to the cell. The appearance of koilocytes on a biopsy from the lower genital tract is highly suggestive of human papillomavirus infection.

Although koilocytosis is still taken by many to be pathognomonic of HPV infection, studies relying on this feature may be misleading. With the advent of more sensitive molecular biological techniques e.g. PCR, it has been shown that this feature does not accurately allow the clear distinction of the subset of HPV positive VIN lesions (Haefner *et al.*, 1995).

The understanding of DNA structure provided the opportunity for the development of more sensitive and specific methods of HPV detection. In the Southern hybridisation technique, crude or extracted DNA is transferred to a membrane and probed with HPV specific probes, whilst DNA:DNA *in situ* hybridisation provided a means to directly demonstrate viral DNA in clinical tissue specimens. However, both these techniques demand the use of type-specific probes and studies were therefore often limited to the detection of only

a few virus types. Nevertheless, early investigation of VIN still demonstrated a high rate (75%) of HPV infection in VIN. If analysis was limited to VIN 3 lesions this rate increased further to 84% (Buscema *et al.*, 1988).

For many years the preferred technique to detect HPV infection has been the polymerase chain reaction or PCR (Mullis *et al.*, 1987; Saiki *et al.*, 1988). This technique is associated with a much greater sensitivity than those already detailed (Table 1-4).

Technique	*Approximate sensitivity*
In situ hybridisation	>25 viral copies / cell
Southern blot	>1 viral copies / 100 cells
Polymerase chain reation	>10 viral copies / 10^6 cells

Table 1-4. Comparative sensitivities of HPV detection techniques (Nuovo, 1990)

PCR primers can be constructed using low annealing temperatures and degenerate base sites to permit mismatch pairing of bases, thereby enabling one set of primers to amplify multiple different HPV types. Two main degenerate primer systems are currently in use for genital HPV typing, namely the MY09/11 primers (Manos *et al.*, 1989) and GP5+/6+ primers (de Roda Husman *et al.*, 1995). Both target regions within the L1 open reading frame (ORF) of genital HPV types (section 1.2.1.2). The MY09/11 system was developed first and amplifies a region of ~450 base pairs, enabling the detection of <100 copies of the HPV genome for a broad range of genital HPV types. The GP5+/6+ system amplifies a shorter ~150 base sequence, localised within the sequence amplified by the MY09/11 primers. Both primer sets have been widely used in genital HPV identification and typing and have been shown to be both reliable and reproducible (Jacobs *et al.*, 1999). The GP5+/6+ system has a potential advantage in that reducing the size of the target sequence should lead to improved sensitivity. This may be of particular importance if the target DNA has been poorly preserved following formalin fixation. A recently designed PCR system (short-fragment PCR, or SF-PCR) has taken this strategy even further, utilising a target of only 66 bases (Kleter *et al.*, 1999). An alternative

means to increase the sensitivity for HPV detection is the use of a two-step nested-PCR. The design of the MY09/11 and GP5+/6+ primers allows for a nested reaction of MY09/11 followed by GP5+/6+ and a single tube real-time system using these pairs has been described (Strauss *et al.*, 2000). The problem of contamination (common to all PCR systems) is of particular relevance when using nested-PCR.

The identification of specific HPV types amplified by degenerate PCR systems can be achieved in a variety of ways including: direct sequencing (Smits *et al.*, 1992); restriction-enzyme digestion (Adams *et al.*, 1996); type-specific PCR (van den Brule *et al.*, 1990) and type-specific oligonucleotide hybridisation (Ylitalo *et al.*, 1995). In this thesis direct sequencing was employed. The sequence obtained from the PCR product was compared against all known HPV sequences available on-line from the Los Alamos National Laboratory Bioscience Division, USA (http://hpv-web.lanl.gov/). Direct sequencing is not limited by the availability of type-specific probes or primers and provides for accurate identification of whichever HPV type has been amplified. However, the alternative methods have the advantage of facilitating detection of infection with multiple HPV types. Sequencing the PCR product will miss such infections as the sequence obtained represents the predominant PCR product present following the reaction. An attractive option for typing multiple infections is the reverse line blot method. In this technique the biotinylated PCR product is hybridised to an array of immobilised type specific probes, before detecting bound probe with a streptavidin-conjugated enzyme (Gravitt *et al.*, 1998). A recent development combines this technique with the SF-PCR described earlier, permitting the simultaneous detection of 16 different HPV types from the 22 base inter-primer region of the PCR product. Although not widely available, this technology is extremely promising. The application of microarray technology to HPV typing may also be of relevance (Cho *et al.*, 2003).

A commercially available alternative to PCR detection is the Hybrid-capture II system (HCII). HCII is based on chemiluminescent detection of hybrids, between RNA probes and target DNA that have been antibody-captured

(Clavel *et al.*, 1998). HCII differentially detects a group of 13 high-risk and 5 low-risk HPV. Precise viral typing is not possible, although contamination is less of a problem than with PCR. The HCII system does however require test specific equipment.

1.1.4.1.2 HPV infection and VIN: PCR-based and immunological evidence

Estimates of the prevalence of HPV infection in high-grade VIN based on PCR methodology vary between 72 and 100% (Haefner *et al.*, 1995; Hørding *et al.*, 1995; van Beurden *et al.*, 1995; Madeleine *et al.*, 1997; van Beurden *et al.*, 1998a) and indeed some investigators have claimed that "VIN 3 always reflects underlying HPV infection" (Hørding *et al.*, 1993). The virus also appears to be transcriptionally active with mRNA from the major oncoproteins being detected using reverse-transcriptase PCR (RT-PCR) (van Beurden *et al.*, 1995). Although initially believed that HPV-associated VIN was confined to the 'younger' age group, Haefner and colleagues demonstrated HPV positive cases in the eighth decade of life (Haefner *et al.*, 1995).

The association between VIN and HPV infection is similar to that seen for cervical disease (CIN). There are, however, some important differences. VIN 3 is usually associated with infection by a single HPV type, namely the oncogenic type 16. This type appears to account for ~86% of infections (Haefner *et al.*, 1995; Hørding *et al.*, 1995; van Beurden *et al.*, 1995; van Beurden *et al.*, 1998a; Rosenthal *et al.*, 2001), with HPV types 18, 31 and 33 making up the remainder (Haefner *et al.*, 1995; Hørding *et al.*, 1995; Rosenthal *et al.*, 2001). Infection with multiple HPV types is uncommon in VIN (Hørding *et al.*, 1995; Madeleine *et al.*, 1997). In contrast, studies of cervical disease have demonstrated a wide variety of HPV types and have shown multiple infections to be common (Bosch *et al.*, 1995; Chang *et al.*, 1997; Kleter *et al.*, 1999). It is also interesting to note that one PCR-based study failed to demonstrate infection with the common HPV types in 101 normal vulval biopsies (Hørding *et al.*, 1993). In cervical specimens transient HPV infection often in the absence of dysplasia is a common occurrence, particularly in young women (Ho *et al.*, 1998; Woodman *et al.*, 2001).

Whether these differences reflect variations in the microenvironment or host immune response between cervix and vulva remains to be seen.

Serological evidence from case controls also lends support to the role of HPV infection in the pathogenesis of VIN. Although the low participation rates for studies is a source of potential bias, seropositivity for HPV 16 is associated with an increased risk of VIN with odd ratios of 3.6-13.4 (Hildesheim *et al.*, 1997; Madeleine *et al.*, 1997).

A causal relationship between HPV infection and cervical neoplasia is now accepted (Bosch *et al.*, 2002). Despite the evidence of a strong association between VIN 3 and oncogenic HPV infection, there are currently insufficient data to support HPV infection as a necessary cause of vulval neoplasia.

1.1.4.1.2.1 HPV and 'differentiated VIN'

In contrast to 'classical' VIN, differentiated VIN is rarely associated with HPV infection (Haefner *et al.*, 1995; Yang *et al.*, 2000). The role of HPV in the pathogenesis of neoplasia will be discussed in detail later, but it is interesting to note that this form of VIN is associated with increased suprabasal p53 protein detected by immunohistochemistry (Yang *et al.*, 2000). Such expression may be taken as circumstantial evidence of mutations in the p53 gene, with the resultant production of a mutant p53 protein that is more stable than wild-type p53 (Baas *et al.*, 1994). Inactivation of p53 function is certainly a potentially important step in carcinogesis. *In vitro* studies of cervical carcinoma cell lines suggest that p53 mutations are only found in HPV-negative cells lines, arising as an alternative to the effects of high-risk HPV oncoproteins on p53 (section 1.2.1.4). This evidence would therefore further support differentiated VIN as a separate entity to 'classical' VIN.

1.1.4.2 Other factors

1.1.4.2.1 Other anogenital intraepithelial neoplasia (AGIN)

In a retrospective review of 133 patients with VIN, up to half were found to have coexistent or previous AGIN (Herod *et al.*, 1996). When VIN 3 alone is considered this proportion may increase further to 66% (van Beurden *et al.*,

1995). Such a high incidence of multicentric disease favours the possibility of HPV infection being the common cause for such lesions. Indeed, HPV infection is significantly (p=0.01) more common in multicentric intra-epithelial neoplasia, as opposed to unicentric VIN3 (van Beurden *et al.*, 1995). Infection is not, however, always with the same HPV type. In one fifth of women with multicentric disease, van Beurden and colleagues found differing HPV types at the various affected sites (van Beurden *et al.*, 1998a).

Patients with CIN are also at an increased risk of subsequently developing VIN. Following an analysis of nearly four and a half thousand patients treated for CIN 3 with cold-knife cone excision and reviewed for 18 years, Reich and colleagues estimated that VIN may develop in 0.3% of cases (Reich *et al.*, 2001).

1.1.4.2.2 Immunosuppression

The increasing incidence of immunosuppression following allogenic organ transplantation has highlighted the influence of host immune response in the genesis of intraepithelial neoplasia of the lower genital tract. Such patients are often younger than usual with multifocal, multicentric disease (Leckie *et al.*, 1977). Idiopathic isolated CD4 cell deficiency can also occur and may be associated with recurrent VIN (Park *et al.*, 1994a). More recently patients with the human immunodeficiency virus (HIV-1) have been shown to have a significantly increased risk of vulval neoplasia (condylomata, intra-epithelial and invasive disease) (Conley *et al.*, 2002). Defects in cellular immune responses may predispose such individuals to HPV infection and subsequent AGIN. The host immune response to HPV will be considered in more detail later in this chapter (section 1.2.2).

1.1.4.2.3 Smoking

The incidence of smoking in association with VIN varies between studies but can be as high as 82% (Modesitt *et al.*, 1998). Jones et al found 60% of VIN 3 patients in their study to be smokers, a figure which was twice the background population rate at the time (Jones *et al.*, 1994). The role of smoking in the aetiology of VIN is supported by case control studies of HPV seroprevalence

(Hildesheim *et al.*, 1997; Madeleine *et al.*, 1997). The effect of smoking on the risk of vulval neoplasia appears to be additive to that of HPV infection (Madeleine *et al.*, 1997).

1.1.5 The presentation of VIN

Most women (~45-65%) with VIN suffer from pruritus or irritation (Bernstein *et al.*, 1983; Barbero *et al.*, 1990; Jones *et al.*, 1994; Herod *et al.*, 1996). The reporting of an atypical area on the vulva is another common presentation, occurring in up to one third of cases (Buscema *et al.*, 1980; Herod *et al.*, 1996). It is important to note that up to one fifth of cases may be asymptomatic (Buscema *et al.*, 1980; Bernstein *et al.*, 1983; Chafe *et al.*, 1988) and are diagnosed by chance during clinical examination for another purpose, e.g. at the time of cervical screening. The high incidence of multicentric disease means that particular attention should be paid to those patients referred for the assessment of intraepithelial neoplasia at other sites. Many patients are embarrassed to discuss genital symptoms and a delay in presentation to an appropriate clinic is commonplace. In their small study of 65 patients, Bernstein *et al.* found that 40% of patients had had symptoms for ≥ 2 years (Bernstein *et al.*, 1983). The reticence of patients is not the only cause for delay in diagnosis and appropriate treatment. Almost three-quarters of women may have seen more than one physician prior to an appropriate diagnosis being made (Bernstein *et al.*, 1983) and 18% of patients wait more than 6 months for referral to a specialist (Jones *et al.*, 1994). Many vulval lesions are treated without a histological diagnosis being made e.g. condylomata, lichen sclerosus. VIN should be considered where such lesions fail to respond to conventional therapy.

1.1.6 Making the diagnosis of VIN

1.1.6.1 Vulvoscopy

Inspection of the vulva under magnification (vulvoscopy) is the cornerstone of diagnosis for VIN. Almost one half of cases will show white lesions on the vulva (Barbero *et al.*, 1990) but in practice the appearance of VIN is extremely variable. Red, pigmented, pale, flat, raised, warty or eroded lesions have all

been described (Ridley *et al.*, 1992). The application of 5% acetic acid to the vulva has been advocated in an attempt to improve detection of VIN lesions. In contrast to the cervix, application of acetic acid to the vulva must be prolonged - for as long as 5 minutes - and is probably more useful for those lesions lacking in surface keratin. The incidence of acetowhite lesions in cases of VIN varies considerably (10-40%) between studies (Barbero *et al.*, 1990; Herod *et al.*, 1996). Care must be taken when using this technique both as many patients with soreness/fissures may find the acid extremely uncomfortable and because the technique has limitations in its specificity. In a study of 40 healthy female volunteers, it was noted that the vestibule became acetowhite in all cases (van Beurden *et al.*, 1997). Twelve patients showed acetowhite change outside the vestibule. These areas showed a weak association with HPV DNA as detected by PCR ($p=0.055$) but were otherwise normal. Failure to appreciate this lack of specificity may lead to unnecessary and uncomfortable biopsies being performed.

When considering the diagnosis of VIN it is helpful to remember that approximately two-thirds of cases have lesions that affect non-hair bearing skin and a similar proportion are unifocal (I.S.G.V.D., 1996). Multifocal lesions are more common in young ($\leq$ 40years) patients (Bernstein *et al.*, 1983) in whom the disease is often associated with HPV infection. The posterior third of the vulva is commonly affected, with the inferior border of the right labium majus reported as the most frequent site for VIN (Bernstein *et al.*, 1983). Particular care should be taken when inspecting the periurethral and perianal regions. Failure to recognise and/or treat disease in these areas may account for the apparent excess risk of progression to malignancy reported for these sites (Jones *et al.*, 1994).

1.1.6.2 Outpatient biopsy

At present, a suspicious lesion identified at vulvoscopy will usually prompt a biopsy from the attendant clinician. The vast majority of specimens are obtained under local anaesthesia with a punch biopsy (illustrated overleaf).

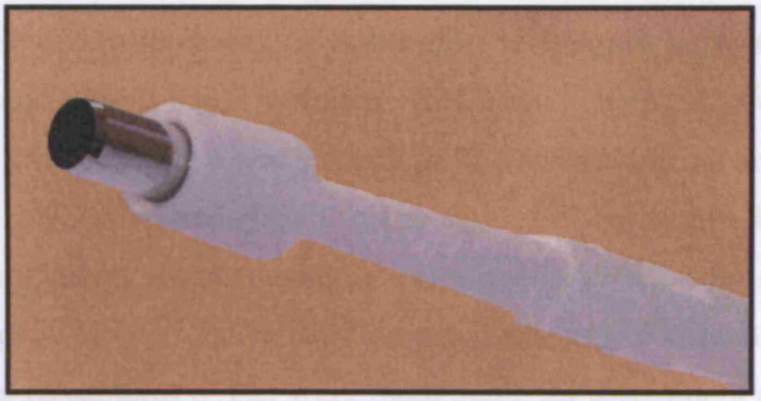


Figure 1-5. Keyes punch used to obtain vulval biopsy. Circular cutting punch available in a variety of sizes but usually used at 4-5mm diameter. After infiltration with local anaesthetic, punch is inserted perpendicular to lesion and with rotating motion. Punch does not need to be used to full depth, as biopsy only needs to include epidermis and dermo-epidermal junction.

Local anaesthesia can be achieved by infiltration, often using a mixture of local anaesthetic and vasoconstrictive agents. The use of topical anaesthetics (such as EMLA cream) alone is rarely sufficient to allow the biopsy to be performed, but makes infiltration of this well-innervated area far more acceptable to the patient. Although this technique has largely replaced excision biopsy in the initial diagnosis of vulval neoplasia, care must still be taken when clinically suspicious lesions are subsequently found to be histologically negative on punch biopsy. False negative biopsies of frankly invasive lesions have been reported (Crawford *et al.*, 1995) and excisional biopsy should therefore be considered where there is a marked discrepancy between clinical and histological specimens obtained at punch biopsy.

1.1.6.3 Alternative techniques for the diagnosis of vulval neoplasia

1.1.6.3.1 Vulval cytology

The potential to obtain a diagnosis without biopsy is attractive to all clinicians who look after women with vulval disease. However, the role of vulval cytology remains controversial. Whilst undoubtedly a successful tool for detecting premalignancy of the uterine cervix, increased keratinisation of the

vulval skin makes obtaining a representative sample more difficult than at other sites. New developments in cytological techniques such as the use of cytobrushes and liquid-based cytology (LBC) may help to overcome such difficulties in the future. In a small study of 23 patients, Levine and colleagues (Levine *et al.*, 2001) prepared Papanicolaou stained slides from LBC specimens obtained from vulval lesions using a cytobrush. All subjects subsequently underwent vulval biopsy. Vulval LBC detected all cases of known dyskaryosis. However, 2 of 28 samples (7.1%) were inadequate for assessment and over one third of dyskaryotic lesions differed by more than one grade from the subsequent histological specimens. Interestingly, the two inadequate specimens were from patients with lichen sclerosus and lichen simplex, two conditions that are frequent differential diagnoses in suspected cases of VIN. For cytology to have an impact on current clinical practice the negative predictive value of a 'vulval smear' will have to improve.

1.1.6.3.2 5-aminolevulinic acid

Photodynamic therapy is commonly used in the field of dermatology and has recently been described as a treatment for VIN (section 1.1.7.3.2). It involves the use of 'photosensitising agents', which may either be applied topically or administered systemically. The agent of choice is 5-aminolevulinic acid (5-ALA), which induces the production of protoporphyrin IX, a precursor of porphyrin. Optimum ratios of photosensitiser in tumour versus normal tissue are achieved at approximately four hours post administration (independent of the route of administration). When viewed under blue light (380-440nm), neoplastic tissue exhibits pronounced red fluorescence, which may enable enhanced detection and localisation of neoplastic disease. Indeed, for the detection of bladder cancer, this technique appears to be more sensitive that conventional white light cystoscopy (Koenig *et al.*, 1999). This concept has undergone limited evaluation for vulval neoplasia. Fluorescence is not exclusive to areas of vulval neoplasia and is also seen in normal introital skin as well as the distal vagina when observed 4-5 hours after topical application (Hillemanns *et al.*, 2000). The accurate identification of VIN 3 is still possible

when the interval post-application of 5-ALA is reduced to 2-3 hours (Fehr *et al.*, 2001) and this may help to reduce such 'false-positive' background fluorescence. Pigmented or hyperkeratotic lesions may not be suitable for assessment by this technique as they are associated with reduced levels of fluorescence (Hillemanns *et al.*, 2000). More recently, oral 5-ALA has been evaluated in the assessment/treatment of cervical neoplasia (Duska *et al.*, 2002). This route of administration has potential advantages with regard to cost and patient convenience but has yet to be studied in vulval disease.

1.1.6.3.3 Toluidine blue test

Toluidine blue is a nuclear stain that has been used to allow the detection of nuclei at the skin surface. In normal vulval skin the surface cells lack nuclei, whilst in VIN 3 surface cells would be expected to have retained their nuclei. The use of Toluidine blue in the identification of vulval neoplasia is not a new technique (Collins *et al.*, 1966; Broen *et al.*, 1971). Recently, a small retrospective study has suggested that the technique may be of particular use in distinguishing VIN from NNED (Joura *et al.*, 1998). However, the technique requires the use of a subjective classification of the staining pattern and, in order to achieve clinically acceptable sensitivities for neoplastic disease, the positive predictive value may be as low as 24% (Joura *et al.*, 1998). The Toluidine blue test is not in widespread use in current clinical practice.

1.1.6.3.4 Serum tumour markers

Squamous cell carcinoma antigen is a glycoprotein with a molecular weight of approximately 45kDa that was originally identified as a possible tumour marker in cervical SCC (Kato *et al.*, 1977). SCC antigen may be useful in the follow-up of patients with vulval cancer, but the overlap in values between VIN cases and the normal population means that this marker is of no clinical use for pre-invasive disease (Hefler *et al.*, 1999). Tissue polypeptide antigen has also been investigated with similar findings (Hefler *et al.*, 2000). Although an attractive goal, a reliable serum marker for pre-invasive disease has yet to be established.

1.1.7 Current treatment options for VIN

VIN presents a therapeutic challenge and a wide variety of medical and surgical treatments have been attempted.

1.1.7.1 Surgical management – excisional techniques

In the UK the principle management of high-grade disease is surgical. Initially, such treatment was extensive, with skinning vulvectomy and skin-grafting advocated for multifocal *in situ* disease (Rutledge *et al.*, 1968). The principle behind this technique is to excise multifocal disease, replacing the 'vulnerable' vulval skin with skin from an ectopic donor site. However, in some individuals even vulvectomy fails to prevent the subsequent development of invasive disease (Hørding *et al.*, 1991). Furthermore, both 'skinning' or 'simple' vulvectomy (primary closure) are extensive surgical procedures requiring general or regional anaesthesia. Both techniques have a risk of significant complications, both local (eg. wound breakdown/infection) and systemic (eg. deep vein thrombosis / pulmonary embolism), as well as the prospect of damaging psychosexual morbidity and sexual dysfunction. For these reasons surgical excision is now usually restricted to local superficial excision wherever possible. Guidelines as to the extent of macroscopic surgical margins required are inconsistent. It would seem unreasonable to insist on similar margins to invasive disease (1-2 cm) for preinvasive lesions but standard gynaecological reference texts usually suggest margins of ≥ 5mm (DiSaia *et al.*, 1997). However, even with histologically clear margins, recurrence may occur in almost one fifth of cases (Modesitt *et al.*, 1998) and this risk appears highest for those with multifocal disease. In this study margins of 1-2cm were advocated. Despite adopting this aggressive strategy, histological assessment revealed two-thirds of margins to be involved with disease suggesting that microscopic disease is commonly more extensive than visible acetowhite change at vulvoscopy would suggest.

The major advantage of excisional techniques is the availability of the subsequent specimen for complete histological assessment. As discussed earlier, occult invasion occurs in 15-22% of cases (Chafe *et al.*, 1988; Hørding *et al.*, 1995;

Modesitt *et al.*, 1998; Husseinzadeh *et al.*, 1999; Thuis *et al.*, 2000). Whilst superficial invasion (<1mm, FIGO stage 1A) is readily treated by local excision with appropriate margins, SCC with a depth of invasion of >1mm carries a significant risk of lymph node metastasis and is routinely treated with radical surgery involving wide local excision and inguinofemoral lymph node dissection (Hacker *et al.*, 1984). The proportion of cases where invasion has already passed undetected through this critical threshold appears to be between 33 and 57% (Chafe *et al.*, 1988; Husseinzadeh *et al.*, 1999). Without full histopathological assessment, these cases would have been missed, increasing their chance of 'recurrent' nodal disease with its associated poor long-term prognosis (Simonsen, 1984).

1.1.7.1.1 Biopsy-directed conservative surgical management

Given the high risk of recurrence associated with surgical excision, a more conservative strategy for the surgical treatment of VIN has been advocated (van Beurden *et al.*, 1998b). In this technique lesions are mapped out with multiple punch biopsies and excisional treatment (with a 5mm margin) is only utilised for those areas subsequently diagnosed as VIN 3. There are however concerns regarding this methodology. Firstly, the extensive 'mapping' biopsies used for multifocal disease required general as opposed to local anaesthesia. Secondly, this technique assumes that punch biopsies will exclude the presence of invasion and that the areas chosen for biopsy are representative of the most severe changes to be found within the lesion. Lesions containing areas of occult invasion are more likely to be exophytic/raised (Chafe *et al.*, 1988) and unifocal (Modesitt *et al.*, 1998). It would, however, be difficult to exclude sampling error for larger areas of multifocal disease. A punch biopsy may also provide a false negative histopathological result (Crawford *et al.*, 1995). Furthermore, occult invasion occurred in only 9% of cases in this study, almost half as frequent as the studies mentioned previously (Chafe *et al.*, 1988; Hørding *et al.*, 1995; Modesitt *et al.*, 1998; Husseinzadeh *et al.*, 1999; Thuis *et al.*, 2000). This finding could indicate significant differences in the study population and makes it hard

to apply the conservative management strategies advocated by the authors to patients with VIN in general.

1.1.7.2 Surgical management - Laser ablation

The use of lasers has been suggested for both excisional and ablative treatment of high-grade VIN. Ablative therapy with lasers may be advantageous in that the fine beam size of the carbon-dioxide laser allows treatment to be delivered precisely to the intended area, with minimum effect to the surrounding normal skin. The depth of treatment can also be limited with a resultant reduction of scarring and a potentially better cosmetic result. Vaporisation appears to be well tolerated and small studies have suggested a cure rate after one treatment of ~75% (Hoffman *et al.*, 1992; Sideri *et al.*, 1999). Care must be taken to continue treatment to an appropriate depth of tissue for the area of vulval skin being treated. Early investigation suggested that vaporisation to a depth of 5mm was required (Mene *et al.*, 1985). More recently it has been suggested that such extensive treatment is unnecessary with 3mm depth being sufficient to ensure the treatment of involved skin appendages in ≥ 95% of cases (Baggish *et al.*, 1989). Although it had been thought that even less radical treatment might be appropriate for non-hairy skin (Wright *et al.*, 1987), this study does not support such a stance, as involvement of sebaceous gland ducts to a depth of 1.27mm and deep hair follicle involvement of ≥ 2.4mm were found in macroscopically non-hairy skin. However, over aggressive vaporisation should be avoided as destruction of the dermal layer may result in increased bleeding, pain and delay in healing with scar formation. In particular, it has been recommended that vaporisation of the labia minora should be limited to a depth of 1mm (providing treatment to 1.5 mm due to additional thermal damage) (Baggish *et al.*, 1989). Reduced operative margins of 3-4mm may also be safe for laser therapy (Baggish *et al.*, 1989).

Despite the potential benefits of laser vaporisation as a treatment there are disadvantages: the cost of laser equipment may limit its use and operating staff require additional safety training; recognition of the surgical planes (Reid, 1985) required for effective treatment requires additional surgical training;

vaporisation does not provide a histological specimen and it is therefore mandatory to ensure the area to be treated has been adequately biopsied prior to ablative treatment; and finally, there may be an increased risk of recurrent disease associated with ablative, as opposed to excisional, treatment (Herod *et al.*, 1996).

1.1.7.2.1 Cavitronic surgical aspiration

Cavitronic surgical aspiration (CUSA) uses the properties of ultrasound to achieve tissue destruction. At an appropriate frequency ultrasound induces cavitations and disruption of tissue, which can then be aspirated, providing a specimen for histopathological assessment. Evidence for the use of CUSA in the treatment of VIN is limited. One study of 37 patients reported 'excellent' long term functional and cosmetic results. However, CUSA for VIN requires regional or general anaesthesia and has only been used to treat to a depth of 1.5mm, which will be insufficient in some cases due to the involvement of skin appendages already described (Baggish *et al.*, 1989).

1.1.7.3 Alternatives to surgery

1.1.7.3.1 Topical Chemotherapy – 5 Fluorouracil

Topical 5-Fluorouracil (5-FU) has been used in the treatment of AGIN. 5-FU is a pyrimidine analogue that functions as a competitive inhibitor of DNA synthesis, blocking the conversion of uracil deoxyribonucleotide to thymidine deoxyribonucleotide. An additional mode of action is direct interference on RNA synthesis and function. Topical 5-FU usually produces erythema and oedema within 48 hours followed by necrosis and ulceration of neoplastic tissues with prolonged use. Neoplastic skin is preferentially affected but normal skin will still demonstrate inflammatory changes. Limited penetration of the drug means that its effects are only reliable in the upper 2mm of skin (Klostermann, 1970).

Although the use of topical 5-FU is potentially attractive in that treatment may be patient-led and healing of the treated areas is usually complete and without scarring, its use on the vulva is not ideal. The keratinised nature of the vulval

skin and the depth to which skin appendage involvement may occur mean that treatment of VIN with 5-FU may well be ineffective. Furthermore, the bystander effect of inflammation of normal adjacent vulval skin frequently limits the dose that can be applied. In their review, Sillman and colleagues report results from seventeen studies (involving a total of 68 patients) where 5-FU was used to treat VIN 3 (Sillman *et al.*, 1985). Remission was achieved in only 34% of cases whilst treatment failed in 59%, usually because patients were unable to tolerate the severe local tissue reaction beyond the first two weeks. The authors propose the alternative of chemosurgery, where a short course of treatment (5% 5-FU applied twice daily for ten days) is followed by physical removal of the partially detached epithelium. This technique has not been widely adopted and data on its efficacy are limited.

1.1.7.3.2 Photodynamic therapy

Photodynamic therapy (PDT) has recently been described as a treatment for VIN. The principle steps of PDT are shown in Figure 1-6. As previously described (section 1.1.6.3.2) the photosensitising agent of choice in the treatment of VIN is 5-ALA, which induces the accumulation of the photosensitiser protoporphyrin IX preferentially in neoplastic tissue. Four hours after topical application the neoplastic areas exhibit red fluorescence under 'blue light' and can be treated by applying non-thermal light with a spectrum that includes 635nm, usually generated by a laser. Irradiation of porphyrin-sensitised skin causes erythema and is accompanied by burning and pain, which may last for hours after therapy. Crusting of the skin is common and the treated area usually heals with 10-14 days. Experience of PDT in the treatment of VIN is limited but disease clearance is reported to occur in 37-53% of treated women (Martin-Hirsch *et al.*, 1998; Hillemanns *et al.*, 2000; Fehr *et al.*, 2001). Unfortunately, the response rate for multifocal high-grade VIN may be as low as 27% (Hillemanns *et al.*, 2000). In addition, a small study found that PDT was unsuccessful in treating of six cases of VIN 3 that had proved resistant to other treatment modalities (Kurwa *et al.*, 2000) and PDT is of limited efficacy in the treatment of hyperkeratotic or pigmented lesions (Hillemanns *et al.*, 2000). The

optimum PDT regime for VIN has yet to be established but it would appear unlikely that PDT will solve the problem of treating this difficult disease.

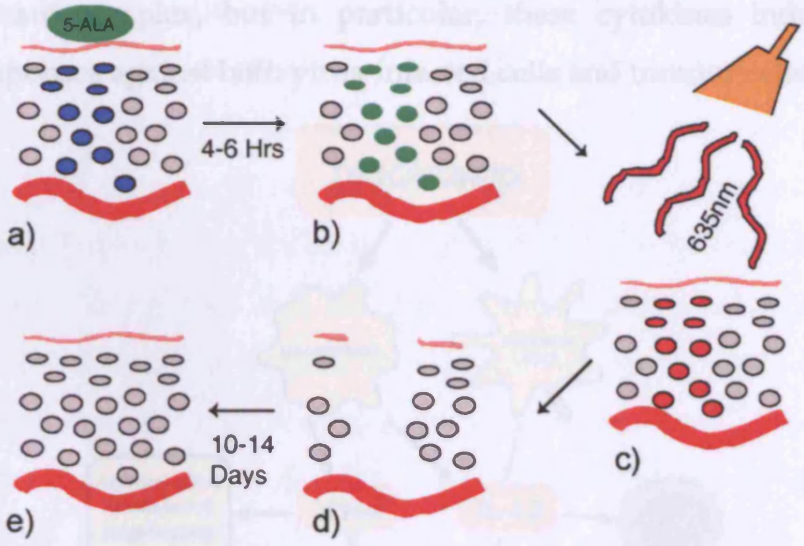


Figure 1-6. Photodynamic therapy. a) Photosensitising agent may be applied topically or given systemically. **b**) Preferential accumulation of agent in dysplastic cells over the subsequent 4 hours **c)** Irradiation with non-thermal light induces of porphyrins with **d)** subsequent destruction of dysplastic epithelium. **e)** As tissue damage is limited to epithelium, healing without scarring usually occurs over the next 10-14 days.

1.1.7.3.3 Topical immunotherapy

Boosting local immunity in an attempt to induce cytotoxic cellular activity against dysplastic cells (often infected by HPV) is a reasonable strategy for immunocompetent patients. A prospective randomised trial of topical interferon-α (IFNα) for the treatment of high-grade VIN demonstrated a complete response, lasting one year, in nine of eighteen patients (50%) (Spirtos *et al.*, 1990). Given this encouraging early evidence it is therefore both disappointing and surprising that there are no other data available regarding this potential therapy.

More recently a novel topical immune modulator been employed to treat high-grade VIN. Imiquimod is an imidazoquinoline that binds the toll-like receptor TLR-7, activating the nuclear factor kappa-B (Hemmi *et al.*, 2002) with the

resultant production of inflammatory cytokines including IFNα and interleukin-12 (IL-12) (Wagner *et al.*, 1999). The consequences of cytokine production are complex, but in particular, these cytokines induce cellular immune responses against both virus-infected cells and tumour cells.

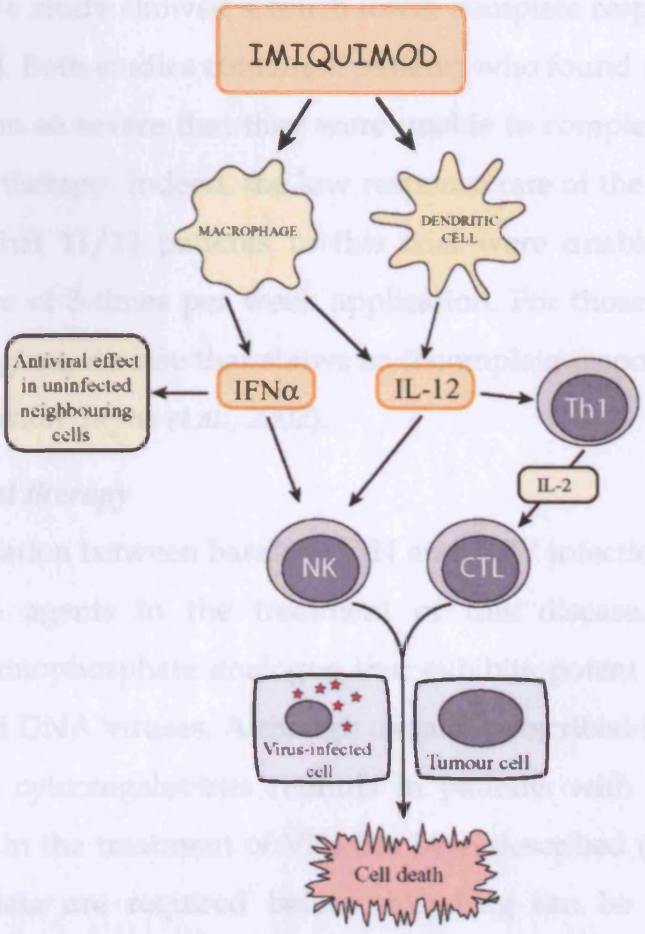


Figure 1-7. Major routes of action of Imiquimod. Imiquimod acts on macrophages and dendritic cells with resultant production of cytokines. IL-12 induces both T-helper 1 cells (Th1) and natural killer cell (NK) activation. Th1 cells subsequently produce interleukin-2 (IL-2) which in turn activates cytoxic T-cells (CTL). In addition to the direct activation of NK, IFNa appears to have a protective effect against subsequent viral infection. Both NK and CTL are involved in cell-mediated immune responses against both tumour and virally infected epithelial cells.

Initial success with Imiquimod in the treatment of condyloma accuminata (Tyring *et al.*, 1998) prompted case reports suggesting that the compound might be of use in the treatment of VIN (Davis *et al.*, 2000; Petrow *et al.*, 2001). These

early reports have subsequently been followed by two small studies of high-grade VIN treated with 5% Imiquimod used in a 3-times per week regime for 12-16 weeks. Whilst a retrospective review of 13 patients suggested that a complete response rate of 62% was possible (Jayne *et al.*, 2002), a similar sized (n=15) prospective study showed a much lower complete response rate of 20% (Todd *et al.*, 2002). Both studies contained patients who found the side effects of local inflammation so severe that they were unable to complete the prescribed course of topical therapy. Indeed, the low response rate of the latter study may reflect the fact that 11/13 patients in this trial were unable to tolerate the prescribed regime of 3-times per week application. For those who are able to tolerate such a regime, disease that shows an incomplete response may indicate foci of occult invasion (Jayne *et al.*, 2002).

1.1.7.3.4 Antiviral therapy

The strong association between basaloid VIN and HPV infection has lead to the trial of antiviral agents in the treatment of this disease. Cidofovir is a deoxycytidine monophosphate analogue that exhibits potent antiviral activity against a range of DNA viruses. Although usually prescribed intravenously for the treatment of cytomegalovirus retinitis in patients with HIV, the use of topical cidofovir in the treatment of VIN has been described (Koonsaeng *et al.*, 2001). Further data are required before this drug can be considered as a therapeutic option for clinical practice, but its efficacy in the immunocompromised patient is particularly attractive.

The development of an effective HPV-vaccination has been a goal of HPV-researchers for many years. The major drive behind such research has been the global burden of carcinoma of the uterine cervix, which currently kills almost 250,000 women every year (Ferlay *et al.*, 2001), mainly in the developing world. Although cervical screening has been effective for developed countries (Vizcaino *et al.*, 2000), the lack of resources in developing countries makes primary prevention an important goal. Compelling data exist to support the development of prophylactic vaccines and a recent study of a viral-like particle (VLP) vaccine (Koutsky *et al.*, 2002) suggests that an effective prophylactic

vaccine should be available for routine use in the next 5-10 years. However, prophylaxis will not affect those women who are already infected with HPV and there is a pressing need to develop effective therapeutic vaccines. Viral targets for therapeutic vaccination and the immune responses required to treat HPV related pathology will be considered at a later stage of this thesis (Chapter 5). Research on VIN has an important role to play in the development of such vaccines. Unlike CIN, the current treatment options for VIN are rather ineffective and often unpleasant for and/or unacceptable to the patient. The preponderance of HPV 16 infection in high-grade VIN means that therapeutic vaccines may therefore focus on this HPV type alone. Advances in therapeutic HPV vaccination and the use of a novel vaccinia-based therapeutic vaccine in the treatment of VIN will be considered in Chapter 5 of this manuscript.

1.1.7.4 Symptom relief

As ~70% of patients with VIN will suffer pruritus and others soreness or discomfort, symptom control is an important part of the management of this condition. Many patients will have been prescribed topical steroids, either as empirical treatment or for symptom control following a known diagnosis of VIN. Whilst steroids are effective in the relief of itching and soreness, there are certainly concerns regarding the application of an immunosuppressive agent to what is often a virus-associated premalignancy. The association between immunosuppression and the development of high-grade VIN has already been described (section 1.1.4.2.2). Molecular studies have suggested at least one mechanism by which steroid administration may affect immune surveillance of HPV-related tumours. *In vitro* experiments suggest that whilst glucocorticoids lead to upregulation of human leucocyte antigen (HLA) class I molecules in HPV-negative cells, the exact opposite occurs in HPV-positive cells. This effect appears to be dependent on viral integration and the transcription of viral genes and can be blocked by the use of steroid receptor antagonists (Bartholomew *et al.*, 1997). HLA class I molecules are crucially involved in the presentation of viral peptides to T-cells and their downregulation following the application of steroids may contribute to immune evasion by HPV. Steroids are therefore

inappropriate in the long-term treatment of VIN. Symptom relief may be obtained by the application of simple agents such as cooled aqueous cream. Silver sulfadiazine (flamazine) has also proved useful as a soothing agent.

1.1.8 Recurrent disease and need for follow-up

The risk of recurrent VIN following treatment may be as high as 50% (Herod *et al.*, 1996; Thuis *et al.*, 2000), and is highest for those with multifocal VIN (Kuppers *et al.*, 1997; Thuis *et al.*, 2000) or multicentric AGIN (Hørding *et al.*, 1995). Pathological grading also appears to be of importance with an increased risk of recurrence evident in those patients with high-grade disease (Kuppers *et al.*, 1997). When excisional treatment is employed, pathological assessment of the margins of the specimen may provide further information as to the likelihood of recurrent VIN. In their study of 73 patients with VIN 3, Modesitt and colleagues found those with histologically involved margins to be at significantly increased risk of disease recurrence when compared to those in whom disease excision appeared complete (46% vs. 17%; p=0.03) (Modesitt *et al.*, 1998). Although this finding was in agreement with an earlier study (Andreasson *et al.*, 1985), other authors have reported high (50%) recurrence rates despite seemingly adequate surgical treatment (Kuppers *et al.*, 1997). Resorting to extensive local excision, such as vulvectomy, fails to prevent recurrent disease and invasive carcinoma may still develop in patients who have undergone this procedure (Hørding *et al.*, 1991). It therefore seems likely that whilst recurrent disease commonly represents persistent or incompletely treated disease, in some individuals new disease does occur. Regular surveillance of patients who have been treated for VIN is therefore essential and most authorities advocate six-monthly to yearly follow-up after treatment. The majority of recurrences will occur in the first four years after treatment (Herod *et al.*, 1996) and it may be possible to safely reduce the frequency of follow-up at this stage. Immunocompromised individuals represent a group at particular risk of recurrence (Park *et al.*, 1994a; Korn *et al.*, 1996). Follow-up for such patients should be long-term at increased frequency e.g. six-monthly for life.

1.2 The multistep process of carcinogenesis in the female lower genital tract

There seems little doubt that the development of carcinoma is usually a multistep process with tumour development occurring as a result of complex interplay between genetic and environmental factors, coupled with the response of the host organism to these stimuli. Three stages in the development of a cancer have been classically described, namely initiation, promotion and progression. Tumour initiation often involves mutational damage to somatic DNA. Subsequent progression is characterised by the expansion of initiated cells with the development of a chronic state of cellular proliferation. At this stage the cells are usually diploid but may show altered differentiation. A benign outgrowth such as a papilloma may then develop. The first stage of tumour progression is the development of a pre-invasive lesion that usually demonstrates further loss of differentiation with aneuploidy and loss of heterozygosity. Lesions that progress further develop an invasive phenotype and commonly demonstrate gene amplification as well as loss of known tumour suppressor genes e.g. p53 mutation.

1.2.1 HPV and lower genital tract neoplasia

The role of HPV infection in the pathogenesis of carcinoma of the uterine cervix has now been clearly established. Persistent infection with HR-HPV is a 'necessary cause of cervical carcinoma' (Bosch *et al.*, 2002) and HPV DNA can be isolated from almost all cases of this cancer using PCR (Walboomers *et al.*, 1999). However, although strong associations have been described, firm evidence establishing causality is lacking for HPV at other anogenital sites.

1.2.1.1 HPV classification

Papillomaviruses are small, double stranded DNA viruses of the papovavirus family, which show both species- and tissue-specificity, infecting only stratified epithelia. Human papillomaviruses also show a degree of site specificity, whereby they produce lesions only at certain anatomical sites. This site-

specificity has led to the division into those HPV types that affect skin (cutaneous HPV) and those that affect mucosal surfaces (mucosal HPV). HPV types are assigned on the basis of the genotype of the virus. Since 1995 this typing has been made on the basis of the L1 gene or open reading frame (ORF) alone. To classify an HPV sequence as representing a new HPV type, the virus sequence should show ≥ 10% difference in the amplified L1 ORF from its most similar known type. More than 130 types have been identified on the basis of the L1 ORF sequence, but the full sequence is currently only available for around 85 of these. Computer analysis of the genetic sequences allowed the construction of phylogenetic trees for HPV. This has shown the genomic classification system to correlate closely with the clinical classification.

The study of cervical cancer has allowed the subdivision of mucosal HPV into high- and low-risk types (HR-HPV and LR-HPV) on the basis of those types most likely to be associated with the development of malignancy and those which are associated with benign lesions such as warts. This classification was originally proposed in the 1980's (zur Hausen, 1986) but a recent study has reaffirmed the validity of such a division (Muñoz *et al.*, 2003). Examples of both groups are shown in the table below.

Mucosal HPV group	*HPV types identified*
High-risk	16,18,31,33,35,39,45,51,52,56,58,59,68,73 and 82
Low-risk	6,11,40,42,43,44,54,61,70,72,81 and CP6108

Table 1-5. High-risk and low-risk HPV types (Muñoz *et al.*, 2003). Types 26,53 and 66 should be classed as probable HR-HPV.

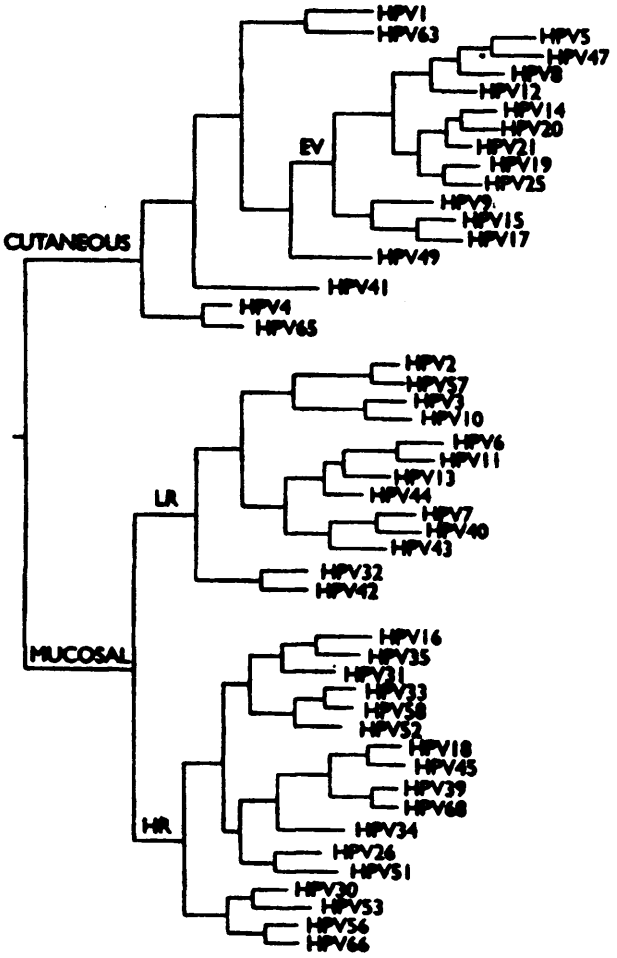


Figure 1-8. Phylogenetic similarities between papillomaviruses. Note subdivision of mucosal types into high and low risk. Reproduced from Van Ranst *et al.*, 1992.

1.2.1.2 HPV structure

The HPV virus particle measures 55nm in diameter and comprises an icosahedral protein coat enveloping the double-stranded DNA (Williams *et al.*, 1961). The genome is approximately 8000 base pairs in length and encodes eight genes. In contrast to other papovaviridae, HPV have all ORF (sequences encoding the genes) arranged on one strand. The genes are divided into early and late genes (denoted by the prefix 'E' or 'L') on the basis of their role in the virus life cycle. In addition to the ORFs, there is a non-coding region (NCR) also known as the long control region (LCR) or upstream regulatory region (URR). This regions varies between 0.5-1kb and is rich in motifs that bind cellular transcription factors (including AP1 and NF1) as well as binding sites for the E1

and E2 proteins that allow for feedback control from viral transcription (O'Connor *et al.*, 1995). The organisation of the HPV 16 genome is shown as an example of a genital HPV type and it is these types that will be considered in further detail within the scope of this thesis.

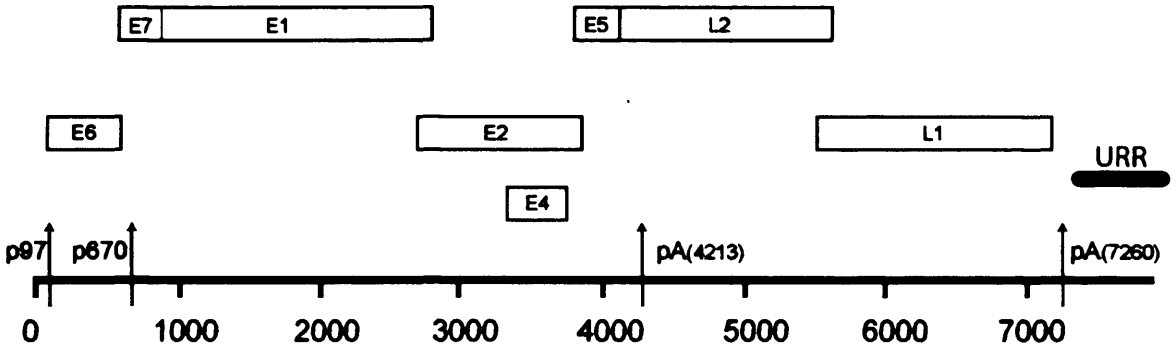


Figure 1-9. Linearised HPV 16 genome showing ORFs. Genome comprises 7905bp arranged in a circular fashion. 'E' denotes early genes whilst 'L' denotes late genes. Positions of early gene promoter (p97) and differentiation dependent late gene promoter (p670) are indicated. The polyadenylation signals for both early and late transcripts are shown (pA). In addition to ORFs the non-coding upstream regulatory region (URR) or long control region (LCR) is shown.

1.2.1.3 Virus life cycle

Human papillomaviruses are thought to enter through 'microlesions' in stratified squamous epithelium. Recent evidence has suggested α6 integrin as a potential receptor for virus entry (Evander *et al.*, 1997). Virus entry may then be followed by a period during which there is no overt evidence of infection. Indeed, studies looking at the possibility of using HPV testing to screen for cervical neoplasia have shown a high incidence of transient infection often with high-risk types, particularly in younger women (Ho *et al.*, 1998). It may be that such individuals undergo abortive sub-clinical infections but the possibility of latent HPV infection must be considered. Many investigators consider the increased incidence of AGIN in immunocompromised patients to represent the

reactivation of latent HPV infection rather than the acquisition of new HPV infection.

HPVs exhibit a differentiation-dependent life cycle, following the keratinocyte in its passage from basal layer towards the surface of the stratified squamous epithelium. Initial infection of the basal epithelium occurs at a level of ~10 copies per cell. The HPV genomes remain episomal (or extra-chromosomal) and copy number is rapidly increased to about 50-100 copies per cell (Stubenrauch *et al.*, 1999). As basal cells divide to replace the more superficial cells, viral genomes are shared between daughter cells. HPV production requires differentiation and does not therefore occur in the basal cells. These cells are therefore spared a potentially lytic viral infection and can remain present as a source of virus for prolonged periods. The need to complete a life cycle in differentiating cells poses a potential problem to HPV. The virus is dependent on the cellular machinery to replicate, but differentiating cells have usually exited the cell cycle. The transcripts from the early ORFs function to overcome this quiescent environment. The E6 and E7 proteins are the major transforming proteins of genital HPV types and serve to induce the differentiated keratinocytes to enter S-phase, whilst at the same time preventing the apoptotic response that is usually associated with inappropriate DNA synthesis in normal cells. The details of this process will be considered in the following section.

Transcription of the early ORFs is initiated from the early promoter located upstream of the E6 ORF - known as p97 in HPV16 (Smotkin *et al.*, 1986). Effective viral replication appears to be dependent on the interplay of several early proteins. Whilst transient viral replication requires only E1 and E2 expression (Del Vecchio *et al.*, 1992), *in vitro* studies using mutant E6 and E7 constructs have demonstrated that these proteins appear to be essential for the maintenance of stable replication in human foreskin keratinocytes (Thomas *et al.*, 1999). The E1 and E2 proteins have a complex interaction with one another and appear to exert a controlling influence on the process of early replication. Whilst E2 is able to undergo high affinity binding to E2-specific sequences in the URR, E1 binds adjacent A/T rich sequences in the replication origins (ori)

with low affinity. Complex formation between E1 and E2 alters both the target and affinity of this binding and may allow for a means of influencing the replication of viral episomes (Frattini *et al.*, 1994).

Full length E2 may cause both transcriptional activation and repression via a variety of different mechanisms (Table 1-6). In a fashion analogous to the oncoproteins E6 and E7, DNA binding and hence transcriptional effects appear to vary between HR- and LR-HPV types, with HPV 16 E2 demonstrating the strongest effects *in vitro* (Hou *et al.*, 2002). The major action of HPV 16 E2 has been thought to be to repress the transcription of E6 and E7 in the basal epithelial cells. However, recent work with cell lines containing both integrated and episomal HPV 16 has suggested that such transcriptional regulation may be a secondary effect, with the main mechanism of E2 control being directly on viral replication itself (Bechtold *et al.*, 2003). Whilst E2 has no enzymatic activity, E1 has been shown to have both ATPase and helicase activity (Hughes *et al.*, 1993). E1 also facilitates viral replication by recruiting cellular replication enzymes such as DNA polymerase α to the viral ori (Park *et al.*, 1994b).

Activation	*Repression*
Recruitment of general transcription machinery	Displacement of cellular transcription factors
Interaction with nuclear factors eg. Sp1	Prevention of pre-initiation complex formation
Remodelling of DNA structure	

Table 1-6. Mechanisms of E2 transcriptional effects (Hou *et al.*, 2002)

As has been stated previously, the virus life cycle is differentiation dependent. As the keratinocyte moves toward the epithelial surface the differentiation dependent promoter (known as p670 in HPV 16 (Grassmann *et al.*, 1996)) is activated with the subsequent expression of HPV late genes. E1 and E2 production are also enhanced following activation of this promoter (Klumpp *et al.*, 1999).The increased levels of these proteins is thought to lead to a marked

increase in viral copy number, at least in part by interference with the usual mitotic checkpoints (Frattini *et al.*, 1997).

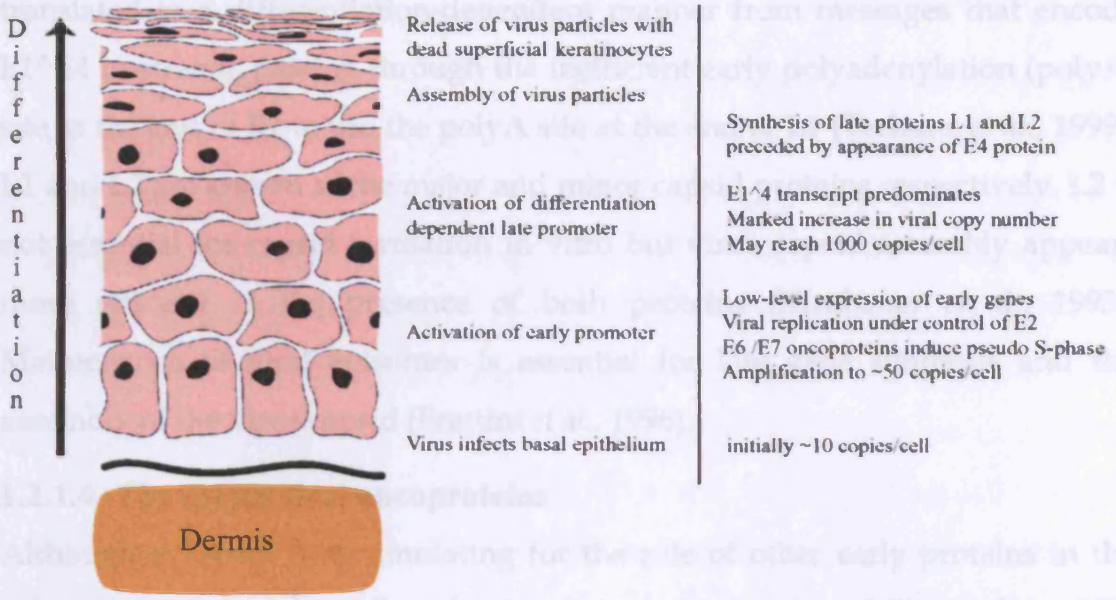


Figure 1-10. Productive HPV infection demonstrates a differentiation dependent virus life cycle

Differential splicing accounts for the production of at least three transcripts from the late promoter. Despite the 'early' prefix, E4 production is largely under the control of this promoter. The major transcript demonstrated in the upper two-thirds of the epithelium is the E1^4 transcript (Iftner *et al.*, 1992). The fusion protein from this transcript is predominantly from E4, with just five amino acids from the C terminus of E1. The protein associates with cellular cytokeratin networks and in cell culture, this association appears to result in collapse of such networks (Doorbar *et al.*, 1991). This process may allude to a role for the E1^E4 fusion protein in preparation for virus exit but more recently it has been shown that the protein provides the virus with yet another way of interfering with the host cell cycle. HPV 16 E1^E4 induces G2 arrest in HeLa cells (Davy *et al.*, 2002). This strategy has been used by other virus e.g. baculovirus, parvovirus and HIV, to enhance virus production in the late stages of the viral life cycle, and may explain the widespread distribution of this protein in the upper epithelial layers described earlier.

In productive HPV infections, the appearance of the E4 protein precedes that of the LI and L2 structural proteins (Doorbar *et al.*, 1997). These proteins are translated in a differentiation-dependent manner from messages that encode E1^E4 upstream, passing through the inefficient early polyadenylation (polyA) site at the end of E1 to use the polyA site at the end of L1 (Terhune *et al.*, 1999). L1 and L2 are known as the major and minor capsid proteins respectively. L2 is not essential for capsid formation in vitro but virus capsid assembly appears more efficient in the presence of both proteins (Kirnbauer *et al.*, 1993). Maintenance of viral episomes is essential for late gene synthesis and the assembly of the virus capsid (Frattini *et al.*, 1996).

1.2.1.4 The major viral oncoproteins

Although evidence is accumulating for the role of other early proteins in the subversion of the host cell cycle, the major transforming abilities of the HR-HPV are thought to be conferred by the E6 and E7 oncoproteins. Together these proteins combine to induce the differentiated keratinocyte into S-phase and allow the cell to avoid the usual apoptotic response to such an event. The major effects of these proteins are discussed below and are summarised in Table 1-9.

1.2.1.4.1 The E7 oncoprotein

The E7 oncoprotein is a small protein of approximately 100 amino acids, which shares structural similarities with both the adenovirus 5 E1A and SV 40 large T oncoproteins. Like these proteins, HR-HPV E7 is able to bind to the retinoblastoma protein (pRb) (Dyson *et al.*, 1989) and associated pocket proteins p107 and p130 (Dyson, 1998). This binding is dependent upon homologous motifs within the amino-terminal of the protein. These proteins all function as regulators of cell growth, at least in part by their interaction with the E2F family of transcription factors. Both phosphorylated and non-phosphorylated forms of pRb exist at various stages of the cell cycle. The non-phosphorylated form of pRb binds E2F. Phosphorylation of pRb by cyclin dependent kinases (CDKs) serves to drive cell cycle progression by releasing E2F. HR-HPV E7 binds non-phosphorylated pRb, p107 and p130, releasing E2F with the resultant increase in E2F inducible genes, including cyclin E (Ohtani *et al.*, 1995) and cyclin A

(Yam *et al.*, 2002). This transcriptional activation allows the cell to overcome both the G1-S and G2-M checkpoints and is a major mechanism by which HR-HPV E7 contributes to oncogenesis.

The carboxy-terminus of HR-HPV E7 contains two cysteine-X-X-cysteine repeats, which have several functions. Acting via this domain HR-HPV E7 can trans-activate the AP-1 family of transcription factors (Antinore *et al.*, 1996), which participate in the transforming effects of E7. Another contribution of this terminus of the oncoprotein is to further encourage cell cycle progression by inhibiting the CDK inhibitors (CKIs) WAF1 (p21) (Funk *et al.*, 1997) and KIP1 (p27) (Zerfass-Thome *et al.*, 1996). Although these inhibitors are themselves induced by E7-mediated increases in E2F, E7 is able to prevent regulation of the cell cycle by directly inhibiting these CKIs. It is worth noting that in productive infections both cyclin E and WAF1 are co-stabilised with KIP1 in some keratinocytes containing HR-HPV E7, apparently as a host mechanism to suppress S-phase entry and viral replication (Noya *et al.*, 2001).

1.2.1.4.2 The E6 oncoprotein

The E6 protein is approximately 150 amino acids in length. A major effect of this protein is to interfere with the function of the cellular tumour suppressor gene (TSG) p53. The p53 protein is induced by a variety of cellular 'stress' signals and acts to protect the cell against inappropriate DNA synthesis by promoting growth arrest (G1-S arrest) and apoptosis. As HPVs rely on cellular DNA replication machinery for their life cycle this protein poses a threat to viral replication. HR-HPV E6 is able to counteract p53 function by both direct and indirect methods. E6 leads directly to the breakdown of p53 by ubiquitin-dependent proteolysis (Scheffner *et al.*, 1990). This interaction is mediated by a cellular protein E6AP (Huibregtse *et al.*, 1991) which functions as a ubiquitin ligase, the E6-E6AP complex targeting p53 for ubiquitination and subsequent degradation. *In vitro* work suggests that a polymorphism at codon 72 (arginine replacing proline) of the p53 TSG may increase the susceptibility of p53 to E6 mediated degradation for individuals homozygous for the arginine allele

(Storey *et al.*, 1998). However, epidemiological studies of HPV-associated neoplasia, including a study of cervical carcinoma patients in the UK (Rosenthal *et al.*, 1998), do not support a role for this polymorphism *in vivo*. Furthermore, in a small study of HPV associated vulval neoplasia, arginine homozygotes were significantly less common in cases as compared to control subjects, suggesting that *in vivo* this polymorphism may actually confer some form of protection against the development of HPV-associated vulval neoplasia (Rosenthal *et al.*, 2000). HR-HPV E6 can also indirectly inhibit p53 action by binding and inhibiting the transcriptional coactivator CBP/p300. In addition to its effects on p53, HR-HPV E6 acts to prevent apoptosis induced by the Bak protein, which is highly expressed in differentiating keratinocytes (Thomas *et al.*, 1998). These two mechanisms are crucial in allowing the infected keratinocyte to escape the normal protective responses to the proliferative signals induced by the E7 oncoprotein.

In addition to its anti-apoptotic effects, HR-HPV E6 activates telomerase. This RNA-dependent DNA polymerase serves to correct the shortening of the telomeres that usually occurs with each round of cell division. Telomeric length is a crucial determinant of cell life. Telomerase activity is necessary for the immortalisation of cells and can be demonstrated in more than 90% of human cancers (Kim *et al.*, 1994). However, telomerase induction alone is insufficient to immortalise human keratinocytes. Once again, it would appear that there is a synergistic effect with the oncoprotein E7, whereby both E7-induced inactivation of the Rb pathway and E6 induction of telomerase are required to achieve the immortalisation of keratinocytes *in vitro* (Kiyono *et al.*, 1998).

Finally, HR-HPV E6 may be able to reduce cell-cycle control effected by the CDKIs. Recent work has shown that HPV 16 E6 can bind the coactivator of the retinoic X receptor alpha (Zeng *et al.*, 2002). This interaction may enhance S-phase progression by reducing transactivation of the CDKI WAF1 (p21).

1.2.1.4.3 The role of HPV E5

Although a major oncoprotein in bovine papillomaviruses (reviewed in DiMaio *et al.*, 2001), the exact role of the E5 protein in HPV infection remains uncertain. *In vitro* work has shown that the protein forms stable complexes with a variety of growth factors (Hwang *et al.*, 1995) and a membrane-bound ATPase which forms part of the gap junction complex (Conrad *et al.*, 1993). These studies provide possible mechanisms by which HPV E5 may contribute to cellular transformation and interfere with normal cellular communication. More recently, E5 from the HR-HPV type 16 has been shown to protect keratinocytes from ultraviolet radiation-induced apoptosis (Zhang *et al.*, 2002). In contrast to the p53 mediated anti-apoptotic effects of HPV 16 E6, E5 achieves this protection at least in part by interference with the caspase family of cysteine proteases. However, early evidence suggested that in cervical carcinomas the gene is frequently deleted or simply not expressed (Schwarz *et al.*, 1985). It is most likely that the role of E5 would therefore be at an early stage of viral infection.

1.2.1.5 The physical state of the virus

The role of viral integration in the development of vulval carcinoma has not been studied but it has been investigated for carcinoma of the cervix. The frequency of integration appears to increase with the severity of cellular dysplasia. In early pre-malignant lesions (LSIL - low grade squamous intra-epithelial lesions) the virus usually exists in its episomal form. As has already been described, this form of the viral genome is essential to allow completion of the virus life cycle with synthesis and assembly of the capsid proteins (Frattini *et al.*, 1996). For more advanced high-grade squamous intraepithelial lesions (HSIL) there have been estimates of the percentage of lesions that contain integrated forms of HPV ranging from 6% to 90% (Daniel *et al.*, 1995; Kalantari *et al.*, 2001). Analysis of cervical SCC has shown the vast majority (90 -100%) of tumours to contain HPV in the integrated form (Park *et al.*, 1997; Kalantari *et al.*, 2001). Integration would appear to have effects on both host and viral gene

expression. Early evidence suggested that disruption of the E2 region during integration resulted in increased expression of the E6 and E7 oncogenes (Schwarz *et al.*, 1985; Cripe *et al.*, 1987) leading to an increased capacity to cause immortalisation and hence malignant progression (Romanczuk *et al.*, 1992).

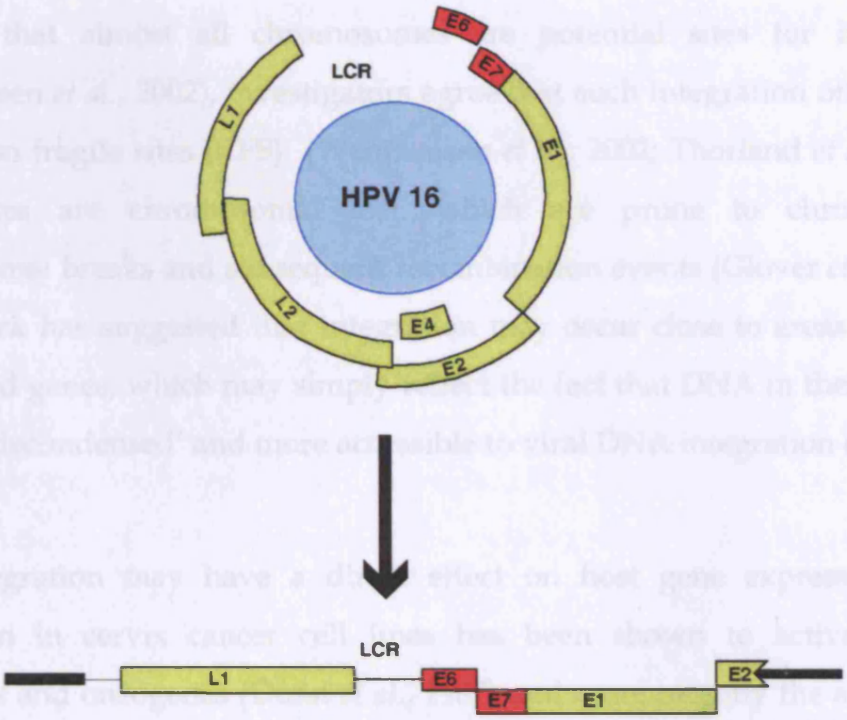


Figure 1-11. Integration of viral genome into host DNA. Integration typically occurs within the E2 ORF, which may reduce the negative transcriptional regulation of the high-risk oncoproteins E6 and E7 (shown in red).

The increase in E6/E7 activity may in part be mediated by an increase in transcript stability due to transcription of 'fused' viral and cellular sequences (Jeon *et al.*, 1995). In carcinomas where the virus remains in its episomal form, mutations in putative binding sites for known cellular transcriptional repressors (such as YY1 (May *et al.*, 1994)) have been shown. This finding supports the hypothesis that it is the loss of E6/E7 transcriptional control following E2 disruption that is the crucial event in developing malignancy following integration. There is however, some evidence to support a direct role for E2 loss in oncogenesis. The HPV 16 E2 protein can induce apoptosis *in vitro* in both HPV-transformed and non-HPV-transformed cell lines (Webster *et al.*, 2000), probably via a p53-dependent pathway. The disruption of the E2 gene at

integration might therefore lead to reduced apoptosis via this pathway, further allowing the E6/E7 stimulated cells to escape the usual control mechanisms for cellular growth.

Although the study of transcripts derived from integrated HPV 16 E6/E7 DNA suggests that almost all chromosomes are potential sites for integration (Wentzensen *et al.*, 2002), investigators agree that such integration often occurs at common fragile sites (CFS) (Wentzensen *et al.*, 2002; Thorland *et al.*, 2003)]. These sites are chromosomal loci, which are prone to chromatid or chromosomal breaks and subsequent recombination events (Glover *et al.*, 1988). Other work has suggested that integration may occur close to areas of highly transcribed genes, which may simply reflect the fact that DNA in these regions remains 'decondensed' and more accessible to viral DNA integration (Klimov *et al.*, 2002).

Viral integration may have a direct effect on host gene expression. HPV integration in cervix cancer cell lines has been shown to activate proto-oncogenes and oncogenes (Dürst *et al.*, 1987) and more recently the application of array technology has identified altered expression of groups of genes including increased expression of the IFN-responsive genes (Alazawi *et al.*, 2002). Viral integration may therefore directly enhance the progression towards malignancy or simply facilitate immune evasion by the dysplastic, virally-infected cells. The further application of new technology and the increased understanding of the human genome may provide more detailed insight into both the site and consequences of viral integration and its role in malignant progression.

1.2.1.6 HPV and vulval carcinoma

Vulval carcinoma is uncommon affecting approximately 800 women in England and Wales every year (incidence 2.4 per 100,000 population). Most carcinomas are squamous cell carcinomas and these can further be classified into keratinising and non-keratinising subtypes. The strong association between HPV infection and undifferentiated VIN has been described in section 1.1.4.1.

However, studies investigating its invasive counterpart have consistently shown a lower incidence of HPV detection, with rates of between 30 and 50% commonly reported. These figures must be interpreted with some caution. Some of the early studies used histological markers or *in situ* hybridisation to detect HPV infection rather than the more recently developed and more sensitive PCR based techniques, which may under-represent the true incidence of HPV infection. Furthermore, the relative infrequency of this cancer makes it hard to draw strong conclusions from the limited number of cases available for analysis. However, it would appear that much of the variance in the rates of HPV infection is likely to reflect the heterogeneous nature of the populations studied. Kurman and colleagues classified one hundred vulval SCC into basaloid, warty and keratinising cell types (Kurman *et al.*, 1993). Keratinising tumours account for approximately two-thirds of vulval SCC. The remainder fit into either basaloid (28%) or warty (7%) subtypes. HPV typing studies, which have used this histological classification, tend to support at least two aetiological subgroups of vulval SCC, namely HPV-related and HPV-independent. The results from two such PCR-based studies are detailed in the following table:

		Incidence of HPV infection		
Study	***Number of subjects***	***Overall***	***Keratinising subtype***	***Basaloid/Warty (non-keratinising) Subtypes***
(Nuovo *et al.*, 1991)	23	30%	0%	70%
(Hørding *et al.*, 1994)	78	31%	4%	81%

Table 1-7. Varying incidence of HPV infection in SCC vulva with histological subtype

Basaloid and warty SCC are more commonly found in younger (<60yrs) women and are associated with undifferentiated VIN of the corresponding subtype in over three-quarters of cases (Kurman *et al.*, 1993). In common with undifferentiated VIN, HPV 16 accounts for the vast majority of HPV infections (88%) associated with SCC of the vulva (Hørding *et al.*, 1994), but HPV 18 (Al-Ghamdi *et al.*, 2002) and HPV 33 (Hørding *et al.*, 1994) infections have also been

reported. The evidence for an increasing incidence of vulval SCC in younger women has already been discussed (section 1.1.2). When women under 40 years of age are considered, the association with undifferentiated VIN (95%) and HPV infection (85%) appears stronger still (Al-Ghamdi *et al.*, 2002).

Whilst HPV may well be responsible for the suggested increase in both VIN and subsequently SCC of the vulva in younger women, there is debate as to the impact of HPV infection on the prognosis in vulval cancer. In their small (n=23) series of vulval SCC, Nuovo and colleagues (Nuovo *et al.*, 1991) found an increased rate of lymph node metastasis with HPV-positive tumours (3/7 vs 4/16). This is in direct contrast to the finding in a Dutch study in which multivariate analysis suggested an improved disease prognosis for HPV-positive tumours (Ansink *et al.*, 1994). Hørding and colleagues were unable to demonstrate any difference in prognosis between HPV-positive and HPV-negatives SCC (Hørding *et al.*, 1993) and it would seem likely that the small numbers of cases available for analysis will make the detection of significant differences in outcome difficult.

Keratinising SCCs of the vulva are usually described as affecting older women and are associated with adjacent differentiated VIN, squamous hyperplasia or epithelial dystrophies such as lichen sclerosus (Hørding *et al.*, 1994). Many feel these precursors are rarely found in association with HPV infection and use this statement to support two entirely distinct aetiologies for vulval carcinoma, one HPV-related and one HPV-independent. Differentiated VIN does indeed show a strong negative association with HPV infection (Haefner *et al.*, 1995). However, when lichen sclerosus is considered the evidence is less convincing. Although studies have shown no HPV in areas of lichen sclerosus associated with carcinoma (Leibowitch *et al.*, 1990; Neill *et al.*, 1990), other authors have found HPV in between 22 and 50% of cases (Kiene *et al.*, 1991; Ansink *et al.*, 1994; Haefner *et al.*, 1995). It would therefore appear that HPV and lichen sclerosus are not mutually exclusive and may act as cofactors in the development of vulval carcinoma.

1.2.2 The immune response to human papillomaviruses

HPV infection of the lower genital tract is a common event in young women (Ho *et al.*, 1998). The vast majority of women do not develop overt clinical disease and this fact, coupled with the transient nature of most infections, suggests a central role for the immune system in the control and clearance of genital HPV infection.

1.2.2.1 The typical response to viral infection

Both humoral and cellular immunity typically have roles in the defence against viral infection. Cell mediated immune responses (CMI) are necessary for the clearance of virally-infected cells and antibody responses help to clear free virus particles and prevent subsequent re-infection by the virus.

Viral antigens are presented to T-lymphocytes by antigen presenting cells (APC) in conjunction with major histocompatibility complex (MHC) molecules. The surface markers CD4 and CD8 define two broad categories of T-cell. CD4+ T-cells recognise exogenous antigen that has been processed by specialised APC and presented on the cell-surface in conjunction with MHC class II molecules (HLA -DP, -DQ and -DR). Activation of CD4+ cells requires a second signal mediated via the interaction of CD80 on the APC and CD28 on the T cell. In contrast CD8+ cells have highly specific receptors that recognise endogenous antigen presented in association with MHC class I antigens. CD4+ cells are further divided into T-helper (Th) 1 and 2 cells. These cells exhibit different cytokine production profiles enabling them to influence different immune effector cells. Th1 cells secrete IFNγ and are able to activate macrophages, natural killer (NK) cells and cytotoxic T-cells involved in CMI. In contrast, Th2 cells secrete IL-4, -5 and -10 which activate B-cells and generate a humoral immune response. Which T-cell response arises may well be determined early on in immune activation at the time of antigen presentation by the APC and will be of crucial importance to the nature and effectiveness of the subsequent immune response.

1.2.2.2 The role of cell-mediated immunity in HPV infection

The precise nature of the immune response to HPV has yet to be established, but it would appear that CMI is central to the effective control of HPV infection. The study of genital warts has shown persistent lesions to be characterised by an absence of immune effector cells, whilst regressing warts show a mononuclear cell infiltrate. The infiltrating lymphocytes are largely CD4+ although CD8+ cells are also present in the epithelium (Coleman *et al.*, 1994). Both morphological data and cytokine profiling suggest that the CD4+ response is Th1 biased. In addition, the fact that individuals in whom CMI is reduced are particularly prone to genital HPV infection supports the central role of CMI in combating HPV infection.

1.2.2.3 HPV-specific immunological responses in AGIN

Studying 'natural' immunity to HPV is essential if effective immunotherapies are to be developed. Once again, the majority of experience has been obtained from the study of cervical disease. Serial biopsies of genital lesions are difficult to obtain and research has therefore focussed on systemic rather than local immune responses to HPV-associated AGIN. The limitations of studying such responses will be considered at a later point in this thesis (sections 1.2.2.3.3 and 5.4)

1.2.2.3.1 Humoral immune responses

HPV 16 infections in young women induce seroconversion after a median time of 8.3 months (Carter *et al.*, 1996). Variations in rates of seropositivity are found for HPV16 DNA positive cancers at different anogenital sites, suggesting that immune responses may vary by cancer site (Carter *et al.*, 2001). Although some women fail to mount a humoral response despite persistent viral DNA, persistent HPV 16 infection (as assessed by PCR based detection of viral DNA) is associated with an increased chance of seroconversion[1]. Comparisons of mucosal versus systemic antibody responses assessed by capsomere-based ELISA suggest that mucosal IgA responses occur over a similar timescale to

[1] Galloway et al. Personal communication, HPV Vaccines and Immunotherapies, July 2003, Cambridge.

serum IgG responses. However, the duration of the IgA response (both in mucosal secretions and serum) is significantly shorter (Onda *et al.*, 2003). This feature of the humoral immune response to HPV must be considered when interpreting the encouraging results of a prophylactic virus-like particle (VLP) vaccination study (Koutsky *et al.*, 2002). In this study, vaccination with an HPV 16 VLP provided protection against both persistent HPV infection and the development of CIN over a median follow-up of 17.4 months. Whilst this duration of follow-up exceeds the mean time for both local and systemic IgA reversion (12.0 and 13.6 months respectively; (Onda *et al.*, 2003)), the long-term benefit for this cohort of women may be less dramatic. It remains to be seen whether therapeutically induced antibody responses will have any effect on the natural history of established HPV infections.

1.2.2.3.2 Cell mediated immunity

Most work to date concerns systemic immunity assessed by testing peripheral blood mononuclear cells (PBMC). Such responses are often small and difficult to detect. The current evidence for Th-cell reactivity in man is somewhat contradictory. In a study of women with CIN, HPV 16 E7 reactivity appeared strongest in those patients with persistent or progressive disease (de Gruijl *et al.*, 1998), possibly reflecting the increased availability of viral antigen in such subjects. In contrast, Kaddish and colleagues report Th reactivity to be enhanced amongst those women who clear HPV infection and in whom CIN regresses (Kadish *et al.*, 1997). Using the sensitive ELISPOT technique (section 2.2.5.3) a more recent study has confirmed the presence of memory Th responses in over 50% of apparently 'healthy' individuals (Welters *et al.*, 2003). It is interesting to note that this study found immune responses predominantly to HPV16 E6 epitopes, with little response detected to HPV16 E7. These findings led the authors to conclude that T-cell reactivity against HPV16 E7 was suboptimal during infection and that IFNγ-producing T-cells directed against HPV16 E6 may offer protection against persistent viral infection and the

development of neoplasia. In a recent update of their data[2], the authors reported a reduced proliferative response in those individuals with CIN or carcinoma when compared to the healthy volunteers. Moreover, the responses that were induced appeared to be deficient in effective cytokine production.

The eradication of virally-infected cells requires cell-mediated cytotoxicity and is dependent on the CD8+ cytotoxic T-cells (CTL) and Natural Killer cells (NK). Following the discovery that peptides binding class I MHC antigens shared common motifs (Falk *et al.*, 1991), HPV16 E6/7 immunostimulatory peptides have been designed for the common HLA-A*0201 haplotype (Ressing *et al.*, 1995), which is found in about 40% of the Caucasian population. Such peptides were then used to demonstrate HPV specific CTL in blood samples (Ressing *et al.*, 1996) and subsequently within cervical tumours and the locoregional lymph nodes from such patients (Evans *et al.*, 1997). Whilst initial work focussed on HPV16 E7 peptides, recent studies have lent further weight to the importance of the HPVE6 CTL response in determining viral persistence and possibly malignant progression. In one such study, an E6 specific CTL response was significantly more likely in women who cleared their HPV16 infection as opposed to those with persistent disease (22/40 vs. 0/9; p=0.003). There was no significant difference when an HPV16 E7 specific response was considered (Nakagawa *et al.*, 2000a). Furthermore, an *in vitro* study of cervical cancer cell lines suggests that these cells may escape a CTL response to E6 by defective antigen presentation, mediated in part by decreased expression of MHC class I molecules and transporter associated with antigen processing (TAP) proteins (Evans *et al.*, 2001).

1.2.2.3.3 Improving the detection of CTL responses

There have been concerns that studies using immunostimulatory peptides may fail to detect memory CTL responses, as the peptides may not be appropriate epitopes for all subjects. A variety of strategies have been developed to improve the detection of HPV-specific CTL. Initial attempts to mimic 'natural' antigen

[2] Van der Burg et al. Personal communication, HPV Vaccines and Immunotherapies, July 2003, Cambridge.

presentation involved stimulation of PBMC using CaSki cells (Evans *et al.*, 1996) or full length proteins expressed using viral vectors (Nimako *et al.*, 1997). More recently sensitive quantitative techniques have been developed which allow HPV-specific CTL responses to be measured directly from clinical blood samples. Tetramers are soluble, fluorogenic MHC-peptide complexes that can be used to stain specific CD8+ cells. Such tetramers have been constructed using HLA-A*0201 and the well characterised, immunogenic HPV16 $E7_{11-20}$ peptide (Youde *et al.*, 2000) and these tetramers were able to detect memory T-cell responses from patients with CIN 3.

An alternative technique is the ELISPOT technique (Figure 1-12). ELISPOT is a microtitre-plate-based enzyme-linked immunoassay used to detect cytokines, commonly IFNγ, produced by antigen specific T-cells following isolation of PBMC from blood and stimulation with the appropriate antigen. By knowing the concentration of PBMC seeded in each well, the concentration of antigen specific T-cells in the blood can be estimated. This technique has been used successfully to show HPV16 E7-directed T-cell immunity in subjects with HPV16 positive cervical lesions (van der Burg *et al.*, 2001b). The quantitative nature of this technique allows the detection of an increased response following therapeutic intervention in a patient with a degree of pre-existing T-cell immunity to HPV.

1.2.2.3.4 Cytokines

The fundamental role of cytokines in the development of an appropriate immune response has already been highlighted (section1.2.2.3.4). Cytokines can be broadly divided into immunostimulatory (type1 eg. IFN, IL-2) or immunoinhibitory (type 2 eg. IL-4, IL-10). Following work suggesting that IL-2 responses were greatest in healthy individuals and decreased with increasing disease severity (Tsukui *et al.*, 1996), Clerici and colleagues went on to suggest that production of immunoinhibitory cytokines was enhanced in those individuals with 'extensive' HPV related disease (Clerici *et al.*, 1997). This pattern of cytokine production might reflect a defective immune response in such individuals. However, a subsequent study found increasing production of

IL-2 with disease persistence or progression (de Gruijl *et al.*, 1998). These results relate to cytokine production from PBMC and a small study of local cytokine production measured from CIN explants cultured in vitro found even more heterogeneity in local cytokine responses (Mota *et al.*, 1999). Undoubtedly the local cytokine milieu will be crucial to the outcome for virus-associated neoplastic lesions. Influencing cytokine production has proved useful in benign viral warts and encouraging early results have been seen with VIN (section 1.1.7.3.3). At present the specific cytokine profile associated with spontaneous regression of HPV-associated AGIN remains uncertain.

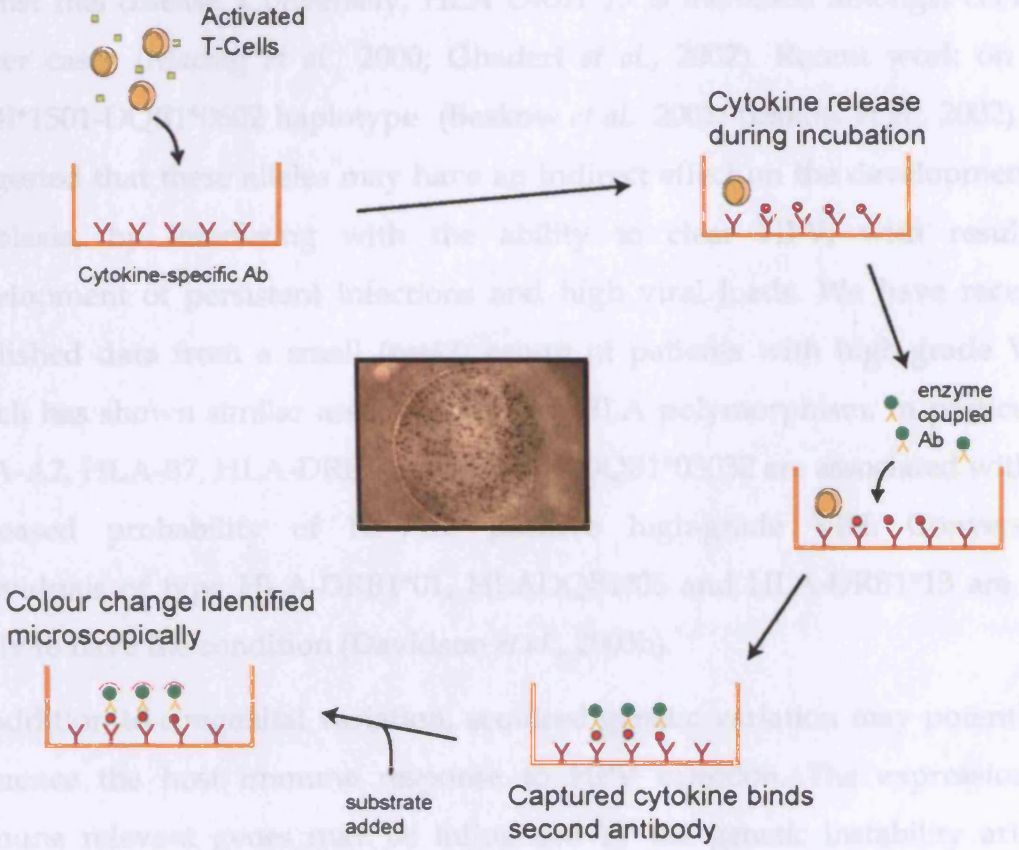


Figure 1-12. The ELISPOT technique. A microtitre plate-based technique to detect cytokine release from activated T-cells isolated from peripheral blood. Central illustration shows typical appearance of 'spots' within well, indicating cytokine production by specifically activated T-cells. Addition of a known quantity of PBMCs allows for a quantitative assessment of the immune response. Control experiments allow establishment of thresholds to allow the identification of both boosted and *de novo* immune responses.

1.2.2.4 Genetic determinants of the host immune response to HPV

HLA polymorphism may have a central role in determining the response to HPV-associated AGIN. For class I MHC molecules, the HLA B*44 genotype has been implicated in disease progression for HPV-positive CIN (Bontkes *et al.*, 1998a), possibly due to specific downregulation of this allele in neoplastic cells (Bontkes *et al.*, 1998b). More recently, population data has suggested a single protective HLA-C allele (Wang *et al.*, 2002). For class two alleles, HLA DRB1*13 has been negatively associated with cervical cancer (Apple *et al.*, 1994; Sastre-Garau *et al.*, 1996; Lin *et al.*, 2001) and may confer some form of protection against this disease. Conversely, HLA DRB1*15 is increased amongst cervical cancer cases (Maciag *et al.*, 2000; Ghaderi *et al.*, 2002). Recent work on the DRBI*1501-DQB1*0602 haplotype (Beskow *et al.*, 2001; Beskow *et al.*, 2002) has suggested that these alleles may have an indirect effect on the development of neoplasia, by interfering with the ability to clear HPV, with resultant development of persistent infections and high viral loads. We have recently published data from a small (n=42) group of patients with high-grade VIN, which has shown similar associations with HLA polymorphism. In particular, HLA-A2, HLA-B7, HLA-DRB1*11 and HLA-DQB1*03032 are associated with an increased probability of HPV16- positive high-grade VIN. Conversely, individuals of type HLA-DRB1*01, HLADQB1*05 and HLA-DRB1*13 are less likely to have the condition (Davidson *et al.*, 2003b).

In addition to congenital variation, acquired genetic variation may potentially influence the host immune response to HPV infection. The expression of immune relevant genes may be influenced by the genetic instability arising from the expression of HR-HPV oncoproteins (section 1.2.3). Furthermore, viral integration may influence the response to anti-viral cytokines such as IFN (Alazawi *et al.*, 2002).

1.2.2.5 Immune evasion by HPV

Our understanding of the immune response to HPV has allowed progress in the development of both prophylactic and therapeutic vaccines (Chapter 5). However, the possible shortcomings of such immunotherapies must be

considered. As a virus, HPV is particularly effective at avoiding an appropriate immune response. This immune evasion accounts for the lag time between primary infection and the development and progression of clinical lesions. Immune evasion is achieved at various different levels within the immune response and some of these mechanisms are outlined in Table 1-8. The success of immunotherapy for HPV-associated AGIN will depend in part on overcoming such immune evasion by the virus.

Level of Immune evasion	*Possible mechanism*	*Comment*
Antigen presentation	Tropism for keratinocytes	- Low levels of HLA molecules may induce non-responsiveness (Bal *et al.*, 1990)
	Antigen threshold	- Murine model shows non-responsiveness at low levels of E7 exposure (Chambers *et al.*, 1994)
	MHC expression	- HPV integration may reduce MHC class I expression (Bartholomew *et al.*, 1997)
	APCs	- Reduced Langerhans cells in dysplasia and HPV infection (Connor *et al.*, 1999)
Cytokines	IL-10	- Increased immunoinhibitory cytokines associated with HPV (Clerici *et al.*, 1997)
	Loss/gain of immune response genes	- HPV-induced genetic instability associated with HPV may alter expression of immune relevant genes - HPV-integration associated with altered expression IFN-responsive genes (Alazawi *et al.*, 2002)
Effector cells	NK inactivation	- In vitro expression of oncogenic E7 blocks NK cell mediated lysis of HPV-transformed cells (Routes *et al.*, 1995)
	Reduced CTL signalling	- CD3 zeta chain expression reduced in CIN and cervical carcinoma. (Kono *et al.*, 1996)

Table 1-8. Possible mechanisms of immune evasion by human papillomaviruses

1.2.3 Genetic events in female lower genital tract malignancy

1.2.3.1 The genetic basis of malignancy

The transformation of normal epithelium to carcinoma occurs with increasing morphological and cellular alterations. In colonic carcinoma it would appear that tumour progression occurs with the progressive and apparently sequential acquisition of genetic change (Fearon *et al.*, 1990). Indeed, genetic alterations are common to many solid tumours and progression is associated with increasing frequency of copy number alterations per case (Ried *et al.*, 1999). The recurrent nature of such changes supports the existence of genes whose functions are central to the development of cancer, namely proto-oncogenes and tumour suppressor genes (TSG). Proto-oncogenes usually promote cell proliferation. Gain of function mutations in cancer cells create versions of these genes with excessive proliferative activity known as oncogenes. Oncogenes behave in a dominant fashion such that mutation of a single allele may affect the phenotype of the cell. In contrast, TSG produce products whose activity limits progression towards carcinoma. Work on familial retinoblastoma led to the proposal of a 'two-hit' hypothesis for TSG, whereby two successive mutations of a TSG are required to turn a normal cell into a cancer cell (Knudson, 1971). An individual who is already heterozygous at a TSG locus (due to a hereditary or acquired mutation) therefore only requires inactivation of the remaining wild-type allele - known as loss of heterozygosity (LOH) - for tumour formation to occur. Mutations that commonly 'activate' oncogenes or TSGs include translocations, point mutations, gene amplifications and gene deletions.

Genetic instability may occur at either chromosomal or nucleotide level (Lengauer *et al.*, 1998). The low rate of spontaneous mutations ($2x10^{-7}$ / cell division) contrasts dramatically with the high incidence of mutations found in tumour cells. This discrepancy has led some authors to postulate the existence of a 'mutator phenotype' in cancer (Loeb, 2001). This phenotype is proposed to arise as a result of mutations in genes whose products that are crucial to the

maintenance of genomic stability e.g. DNA repair enzymes such as polymerases and helicases. However, such a phenotype is not essential as mutations may provide a selective growth advantage with resultant rounds of clonal expansion at exponential rates (Cairns, 1975). At a chromosomal level, aneuploidy may serve to drive genetic instability (so-called 'chromosome error propagation') (Holliday, 1989; Duesberg *et al.*, 1998). Indeed, aneuploidy has been demonstrated in LSIL of the cervix and can therefore be a relatively early step in the development of lower genital tract cancer. Carlson and colleagues used fluorescence *in situ* hybridisation (FISH) to demonstrate a significant progression of chromosome 17 polysomy through the histological spectrum of normal vulvar skin to vulval SCC (Carlson *et al.*, 2000). Normal skin found in association with vulval SCC showed higher rates of chromosome 17 aneusomy than control skin, lending support to the notion of aneuploidy as a driving force at an early stage of vulval carcinogenesis.

1.2.3.1.1 The role of HPV infection in the development of genetic instability

Infection with genital HPV is a common and often transient event in young women (Ho *et al.*, 1998). Only a fraction of infected individuals develop SIL and even when HSIL are considered, only a minority of lesions will progress to invasive disease. Thus, it would appear that HPV infection alone is insufficient for malignant progression. Before an invasive tumour will develop, the normally tight control of viral gene expression (section 1.2.1.3) must be disrupted to allow the expression of early genes in proliferating epithelial cells. Following such disruption, HR-HPV E6 and E7 cooperate to overcome the usual cellular controls on DNA synthesis; to promote aneuploidy and genomic instability; and to prevent the usual apoptotic response to such aberrations (Table 1-9 and section 1.2.1.4).

	High-risk HPV E6	*High-risk HPV E7*
Action	Inhibits p53 function directly by degradation (Scheffner *et al.*, 1990) and indirectly by binding the transcriptional coactivator CBP/p300 (Zimmermann *et al.*, 1999).	Binds and inactivates pRb, p107 and p130 (Dyson, 1998), with resultant increase in E2F inducible genes, including cyclin E (Ohtani *et al.*, 1995) and cyclin A (Yam *et al.*, 2002).
	Inhibits Bak induced apoptosis (Thomas *et al.*, 1998)	Transactivates AP1 transcription factors (Antinore *et al.*, 1996).
	Reduced transactivation of cyclin-dependent kinase inhibitor (CKI) WAF1 (Zeng *et al.*, 2002).	Inhibits CKIs WAF1(Funk *et al.*, 1997) and KIP1(Zerfass-Thome *et al.*, 1996).
	Telomerase activation (Klingelhutz *et al.*, 1996).	Induces abnormal centrosome synthesis (Duensing *et al.*, 2001a).
Net effect	Overcomes G1/S checkpoint Promotes genomic instability (Livingstone *et al.*, 1992) Prevents apoptosis	Overcomes G1-S and G2-M checkpoints. Promotes aneuploidy (Duensing *et al.*, 2001a).

Table 1-9. Contribution of high-risk HPV oncoproteins to genetic abnormalities in lower genital tract malignancy

1.2.3.2 Genetic alterations in squamous cell neoplasia of the vulva and cervix

The combination of a long natural history, well-defined morphology and relative ease of access to tissue specimens lends itself to the study of the role of genetic change in squamous neoplasia of female lower genital tract. Cervical neoplasia is the most common form of lower genital neoplasia and has the additional advantage that well characterised cell lines exist to allow *in vitro* studies to be performed. For this reason, most evidence for recurrent genetic alterations has been gained from the study of CIN and cervical SCC. However, limited data also exist to support recurrent genetic change in both VIN and vulval SCC.

1.2.3.2.1 Classical cytogenetics

Classical cytogenetics involves the study of metaphase chromosomes, which can be identified by their unique banding pattern with certain stains. Karyotype analysis is routinely performed using Giemsa (G-banding) although many other stains have been used e.g. 4,6-Diamidino-2-phenylindole (DAPI). G-banding produces a pattern of three to four hundred alternating light and dark bands along the chromosome length. Giemsa preferentially stains gene poor areas containing repeat-rich sequences (usually A-T repeats) that replicate late during S-Phase. Since its inception in 1960, the International System for Human Cytogenetic Nomenclature (ISCN) has been used to allow the description of a diseased karyotype from the study of metaphase chromosomes. The classification was most recently revised in 1995 (Mitelman, 1995). Although staining techniques allow the identification of alterations ≥ 4000 kb, there are problems with such an approach. Accurate classification will depend on subjective variables such as operator experience. The resolution of staining is crucially dependent on chromosomal condensation and the physical accessibility of the staining agent. Although improved results may be obtained by the use of proteolytic enzymes such as trypsin, the technique is inherently variable. Furthermore, solid tumours often contain sparse metaphases, exhibiting marked aneuploidy and heterogeneity, which are frequently unsuitable for such assessment. Despite these difficulties karyotypic abnormalities have been reported in a small study of six vulval carcinomas (Worsham *et al.*, 1991). Frequent abnormalities seen included both losses (3p, 8p, 22q, Xp) and gains (3q, 11q).

In view of the difficulties in standard karyotype analysis, many early studies focussed on ploidy. In the 1960s aneuploidy was documented in cervical HSIL, then known as carcinoma *in situ* (Spriggs *et al.*, 1962). Cellier *et al.* subsequently defined two groups of HSIL of the cervix, those with a peritetraploid cell population and those with a peridiploid/peritriploid population (Cellier *et al.*, 1970). In this study no invasive carcinoma with an exclusively peritetraploid population was found, leading to the suggestion that only those HSIL with a

peridiploid/peritriploid cell population would progress to malignancy. More recently high rates of aneuploidy have been demonstrated in both vulval SCC (72%) and VIN (80%) (Lerma *et al.*, 1999). The authors' data support an earlier report that used regression analysis to suggest that ploidy was an independent prognostic parameter in vulval carcinoma (Kaern *et al.*, 1992). This view has been challenged by a smaller study of 43 carcinomas, where ploidy status was not found to be of prognostic importance (Ballouk *et al.*, 1993).

1.2.3.2.2 Molecular genetic techniques

Advances in recombinant DNA technology and our understanding of the human genome have allowed investigation of the genetic changes associated with carcinogenesis at a molecular level. Initially genetic polymorphisms were studied by labour intensive restriction enzyme digestion of large amounts of DNA followed by Southern blotting and hybridisation with a radiolabelled probe (restriction fragment length polymorphisms or RFLP). Developments in PCR technology have improved the sensitivity of DNA analysis. The more commonly employed techniques will be described in the following sections.

1.2.3.2.2.1 Loss of heterozygosity studies using microsatellites

Loss of heterozygosity (LOH) studies are normally used to suggest the presence of putative TSG. Microsatellites (MS) are short (1-4 bp) repetitive sequences (often $(CA)_n$) that are interspersed throughout the genome, usually in intergenetic DNA. The length of the repetitive sequence may be highly variable or polymorphic. This feature is used to identify potential TSG by screening paired normal (e.g. blood or stroma) and tumour samples for such sequences. The sequence is amplified using PCR and the products are resolved on an appropriate gel. For a marker to be useful or 'informative', the normal tissue should be heterozygous for the marker polymorphism - indicated by the presence of two bands on the gel, representing alleles of two different lengths. Should a deletion occur within a tumour this will be seen as loss of one of the bands from the tumour specimen - LOH. In practice, many pathological specimens contain a certain amount of normal stroma. As such, LOH often shows as a decrease in the relative intensity of one or other band - also termed

'allelic imbalance' (AI). MS screening for LOH may also suggest the presence of Microsatellite instability (MI), which may reflect the 'mutator phenotype' within an individual tumour. In MI, the gel reveals additional novel bands that are present in the tumour specimen but not in the normal control. Although informative polymorphic MS markers have been identified at approximately 100kb intervals throughout the genome, studying tumours in a global fashion would be a massive undertaking. LOH is therefore usually performed in a more focussed fashion, concentrating on regions of the genome where other evidence has suggested the presence of a potentially important locus. To obtain meaningful results from such studies, ideally a large panel of tumours should be screened with closely spaced MS markers. LOH occurring at a specific locus in ≥ 20 % of cases has been suggested to support the presence of a TSG (Hampton *et al.*, 1994).

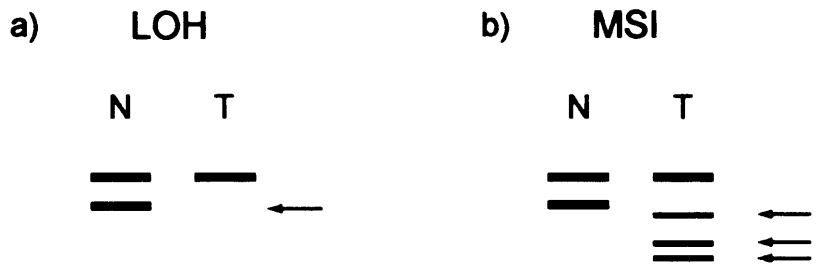


Figure 1-13. Schematic illustration to show loss of heterozygosity (LOH) and microsatellite instability (MSI). Diagram represents bands seen on gel following PCR for an informative MS marker. The normal (N) tissue is heterogygous for the MS marker and therefore shows two products/bands. **a)** LOH is seen as loss of one of the allelic bands in the tumour specimen (missing band indicated by an arrow) **b)** In MSI, the tumour specimen shows additional bands (indicated by arrows) that are distinct from the two alleles seen in the normal specimen.

Recurrent LOH at high incidences has been demonstrated in SCC of the cervix for a variety of loci, including 3p14.1-22, 4p16, 4q21-35, 6p21.3-22, 11p15, and 11q23 (for review see Lazo, 1999). LOH may accumulate in a sequential pattern. Larson and colleagues studied a small group of 42 women with varying grades

of CIN for AI/LOH at selected loci on four of the chromosomal arms identified as commonly affected in cervical cancer. Allelic loss at one or more locus increased in frequency with increasing histology grade of lesion (Larson *et al.*, 1997). A comparison of LOH between invasive carcinomas and concurrent CIN 3 revealed high incidences of LOH at 3p14.1-12 and 6p23, suggesting that these loci may be important early in tumorigenesis (Rader *et al.*, 1998). The concept of progressive loss of genomic material is further supported by data from comparative genomic hybridisation (CGH - section 2.2.4). Specific patterns of allelic loss may influence both prognosis in SCC (Kersemaekers *et al.*, 1998) and disease recurrence following cervical conisation in cases of CIN (Lin *et al.*, 2000).

The first study to document LOH in SCC of the vulva and associated VIN was extremely small preventing any meaningful conclusions from being drawn (Lin *et al.*, 1998). Since this report, several investigators have demonstrated LOH in VIN (Pinto *et al.*, 2000; Rosenthal *et al.*, 2001; Rosenthal *et al.*, 2002) and/or vulval SCC (Flowers *et al.*, 1999; Pinto *et al.*, 1999; Rosenthal *et al.*, 2001; Rosenthal *et al.*, 2002). Indeed, Rosenthal *et al.* found LOH for at least one of the six loci studied to be a common event for both VIN (53%) and vulval SCC (81%). LOH is not unique to neoplasia and can be demonstrated in non-neoplastic vulval dermatoses such as lichen sclerosus (Pinto *et al.*, 2000). However, data from X-chromosome inactivation studies (Tate *et al.*, 1997; Rosenthal *et al.*, 2002) coupled with shared patterns of LOH between SCC and associated VIN (Rosenthal *et al.*, 2002) provides molecular evidence suggesting that VIN is indeed a true precursor lesion for vulval SCC and that the progressive acquisition of genetic damage may be crucial to carcinogenesis. The loci that have been demonstrated to be affected in vulval neoplasia are similar to those found at high prevalence in SCC of the cervix and include 3p, 4q21, 5p14 and 11p15 (Pinto *et al.*, 1999; Rosenthal *et al.*, 2001).

Several known TSG loci have been studied in more detail. Both Flowers and Rosenthal used MS markers to investigate LOH at 13q14, the Rb locus (Flowers *et al.*, 1999; Rosenthal *et al.*, 2001). The authors concluded that LOH for Rb occurred at relatively low frequency and that loss of Rb did not appear to be

important in vulval carcinogenesis. Rosenthal and colleagues also considered the p16 locus (9p21). Whilst LOH at this locus did occur more frequently in VIN associated with carcinoma than VIN occurring in isolation, the fact that the p16 loss occurred at reduced frequencies in the associated carcinomas would suggest that p16 is probably not involved in disease progression (Rosenthal *et al.*, 2001). LOH at the p53 locus (17p13.3) is more common in HPV negative vulval SCC than in HPV positive vulval SCC (Flowers *et al.*, 1999; Rosenthal *et al.*, 2001). As HPV-positive tumours can abrogate p53 function by their high-risk E6 oncoprotein, there is unlikely to be a selection pressure for mutation of the p53 gene within these tumours. Other loci on 3p, 5q, 10p and 10q also show significantly higher rates of LOH in HPV-negative carcinomas (Flowers *et al.*, 1999; Pinto *et al.*, 1999; Rosenthal *et al.*, 2001). These findings may provide an insight into distinct pathways for the development of HPV-negative vulval SCC.

1.2.3.2.2.2 Fluorescence in situ hybridisation

FISH techniques rely on the fact that, under appropriate conditions, a DNA probe will bind to the complementary DNA sequence on a chromosome. The probe can be labelled either directly by incorporation of a fluorescent-labelled nucleotide precursor, or indirectly by incorporation of a reporter molecule (such as biotin or digoxigenin) which can then be detected using a specific, fluorescent-labelled antibody. Large DNA probes are used to improve the intensity of the hybridisation signal and the addition of a large excess of genomic DNA to the probe prior to the hybridisation will saturate repetitive elements within the probe, preventing them from masking the signal generated by unique sequences (Lichter *et al.*, 1990). Molecular genetic techniques based on FISH have improved the resolution of chromosome mapping beyond that afforded by conventional banding analysis and overcome some of the limitations associated with variation in metaphase chromosome preparation (Trask, 1991). Multiplex-FISH, spectral karyotyping and interphase FISH involve the hybridisation of a known probe to target chromosomes in either metaphase spreads or interphase nuclei. Comparative genomic hybridisation

(CGH) reverses this strategy, using a probe constructed from the tumour DNA and hybridising it to normal metaphase chromosomes in competition with normal reference DNA. The technique of CGH will be discussed in detail in the following section.

1.2.3.2.2.3 Comparative genomic hybridisation

CGH permits the rapid and detailed detection of both gains and losses across the whole genome within the confines of a 'single' hybridisation experiment. The technique was first described in the early 1990's (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993) and requires relatively small amounts of DNA which can be obtained from either fresh or archival material. The methodology is illustrated in the following diagram.

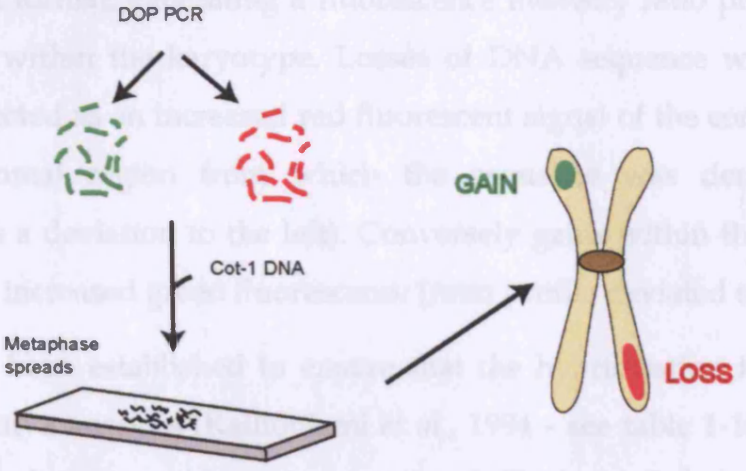


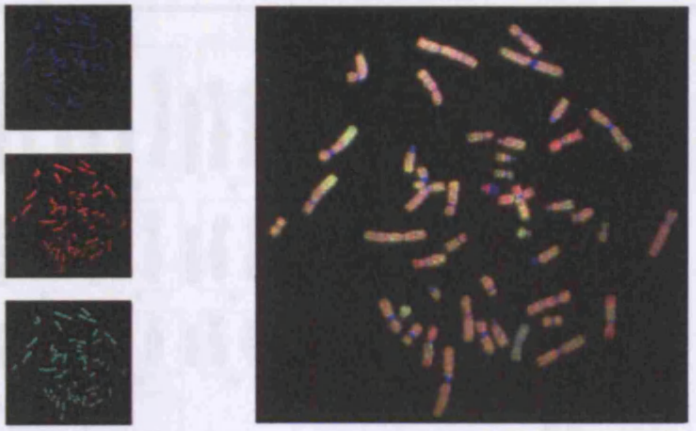
Figure 1-14. Comparative Genomic Hybridisation. DNA from subject and normal reference are differentially labelled (conventionally test DNA is labelled green, control DNA is labelled red). Equal amounts are then used in a competitive hybridisation against normal male metaphase chromosomes on a glass slide. Copy number gains will be indicated by a predominantly green fluorescent signal from that region, whilst losses will give a predominantly red signal.

Equal amounts of 'test' DNA (from the tumour to be investigated) and 'control' normal reference DNA (e.g. WBC from a karyotypically normal individual) are differentially labelled, using either digoxygenin-conjugated or biotinylated nucleotides. The mixture is then used as a probe in a competitive hybridisation

against normal metaphase chromosomes on a glass slide. An excess of repetitive, unlabelled Cot-1 placental DNA is used to suppress the signal from highly repetitive sequences (see above). Following stringency washes the bound DNA is detected by using antibodies directed against the incorporated haptens, each labelled with a different fluorochrome. Conventionally, test DNA is labelled with a green fluorochrome and control DNA with a red fluorochrome. DAPI counterstaining facilitates chromosomal identification. An image of the hybridisation pattern is captured using a fluorescence microscope with a cooled, charged coupled device camera and selective filters and analysed with the aid of appropriate software. The green:red intensity ratio is calculated along the entire axis of each chromosome within a metaphase. The data is converted to a graphical format, generating a fluorescence intensity ratio profile for each chromosome within the karyotype. Losses of DNA sequence within the test DNA are detected as an increased red fluorescent signal of the control DNA on the chromosomal region from which the sequence was derived (shown graphically as a deviation to the left). Conversely gains within the test sample are shown by increased green fluorescence (ratio profile deviated to the right).

Criteria have been established to ensure that the hybridisation is suitable for such quantitative analysis (Kallioniemi *et al.*, 1994 - see table 1-10). Analysing multiple metaphases provides a mean ratio profile for each hybridisation and reduces the possibility of background noise influencing the result. Computerised standardisation of fluorescent signals from each metaphase allows for meaningful comparisons between each experiment. The technique has limits of resolution of 10-12 Mb for deletions (Bentz *et al.*, 1998). The lower limit of detection for copy number increase is dependent on the size of the amplicon and the excess copy number (Piper *et al.*, 1995), but is likely to be between 2-5Mb. Contamination of the tumour sample with normal DNA may mask a real gain or loss and should be minimised by microdissection of the tumour sample where appropriate.

1)



2)

3)

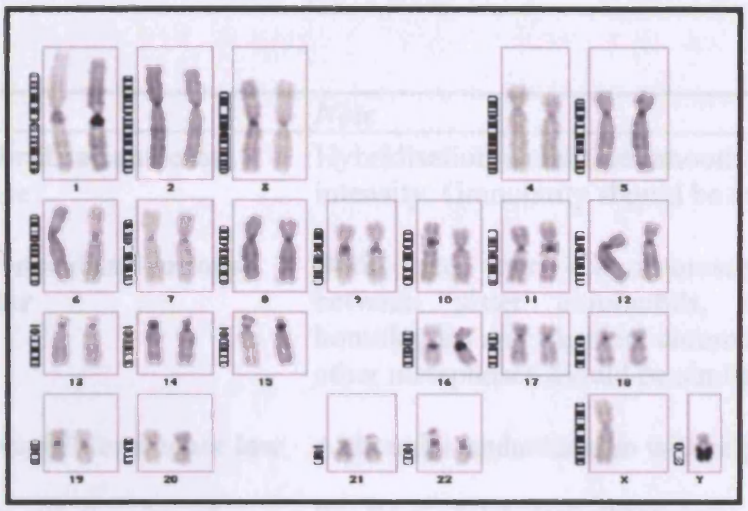
Figure 1-15. Stages of classical CGH analysis

1) Images of suitable metaphases captured using filters to allow visualisation of DAPI staining (blue) and red/green fluorescence respectively. Once captured, a composite image is created.

2) Metaphase displaying DAPI banding pattern

3) Individual chromosomes outlined (blue) to allow sampling of fluorescence ratio along the long axis of the chromosome. Sampling axis shown in green

4)



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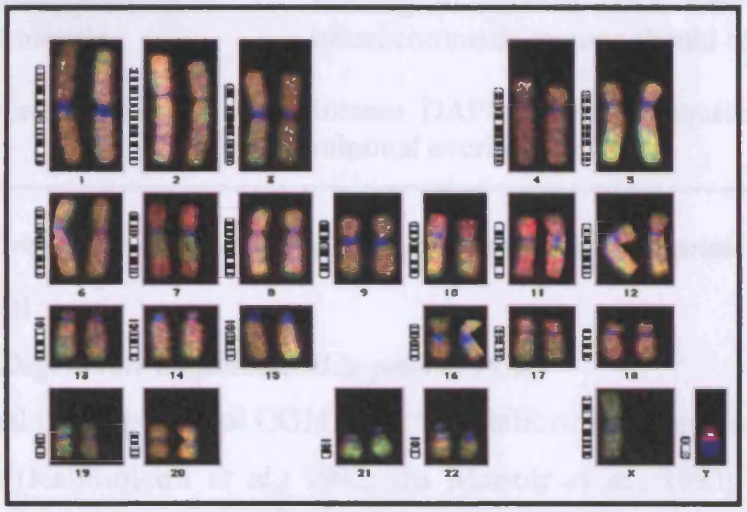

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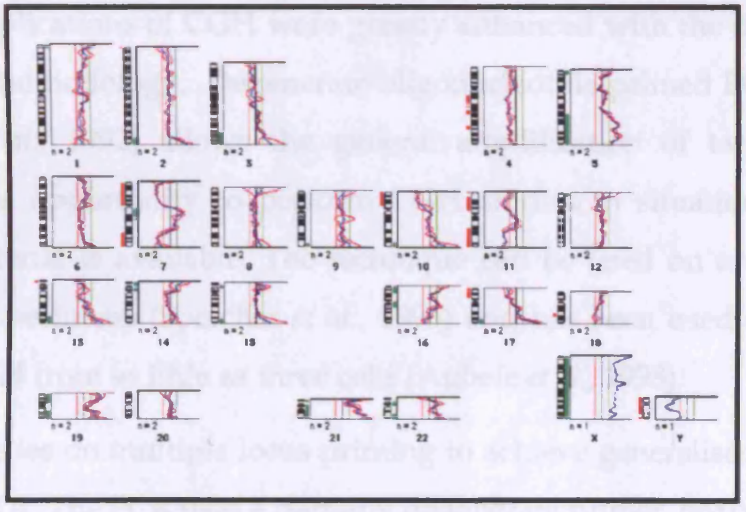


Figure 1-15 cont.) Stages of classical CGH analysis

4) Manual karyotyping performed using DAPI banding pattern

5) Colour image showing chromosome pairs. 'Green' X chromosome and 'red' Y chromosome serve as internal controls for hybridisation reaction.

6) Karyogram showing mean fluorescence ratio profile for each chromosome pair with 95% confidence limits. Threshold for copy number gains (green) and losses (red) set at 1.15 and 0.85 respectively. Process repeated for approximately ten metaphases in each case.

Criteria	*Note*
Even hybridisation across metaphase	Hybridisation should be smooth and of high intensity. Granularity should be avoided.
Fluorescence distributions are similar	Both green and red fluorescence should between sister chromatids, chromosome homologues and identical chromosomes from other metaphases should be similar.
Background fluorescence low	Although standardisation will be performed.
Adequate suppression of heterochromatin	Binding of labelled DNA centromeres and heterochromatic regions should be low.
Appropriate metaphases	Intense DAPI staining, adequate length and minimal overlapping.

Table 1-10. Criteria to ensure quality of hybridisation is appropriate for quantitative analysis of CGH

1.2.3.2.2.3.1 Degenerate oligonucleotide-primed PCR

In the original descriptions of CGH, nick translation was used to create labelled probe DNA (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993). However, the potential applications of CGH were greatly enhanced with the development of novel PCR methodology. Degenerate oligonucleotide-primed PCR (DOP-PCR, Telenius *et al.*, 1992) allows the general amplification of target DNA and provides the opportunity to perform CGH studies in situations where little tumour material is available. The technique can be used on archival paraffin embedded specimens (Speicher *et al.*, 1993) and has been used successfully to perform CGH from as little as three cells (Aubele *et al.*, 1998).

DOP-PCR relies on multiple locus priming to achieve generalised amplification of target DNA. The PCR uses a partially degenerate primer, 6MW (Figure 1-16). Starting at the 5′ end, the primer consists of a standard 3 base linker, followed by a hexanucleotide restriction enzyme site (Xhol) and a degenerate middle section. 6MW relies initially on priming at low annealing temperatures from short sequences at the 3′ end of the oligonucleotides used. These short

sequences will occur frequently and facilitate primer annealing at approximately 4Kb intervals throughout the genomic template. The 3′ end of the primer contains an anti-codon for methionine, followed by glycine. Glycine is believed to be the most frequently occurring amino acid to follow the initiating methionine in eukaryotic proteins and these bases therefore preferentially direct annealing towards gene rich regions of the genome. The consistent 5′ sequence allows for efficient annealing of the primer to previously amplified DNA at higher annealing temperatures used in later PCR cycles.

Figure 1-16. Sequence of 6MW primer used in DOP-PCR

5′ CCG ACT CGA GNN NNN NAT GTG G 3′

N = any base in an equal ratio.

1.2.3.2.2.3.2 CGH studies of vulval and cervical neoplasia

CGH has been applied to a wide variety of human tumours (summarised in (Struski *et al.*, 2002) including tumours at a variety of sites in the female genital tract. Studies of SCC of the cervix have shown a consistent pattern of copy number imbalances (CNI). Common losses have been shown to affect 2q, 3p, 4p, 4q, 5q, 6q, 13q and Xq. Chromosomes demonstrating consistent copy number increases include 1q, 3q, 5p, 6p, 8q, 17p, 17q and 20 (Heselmeyer *et al.*, 1996; Heselmeyer *et al.*, 1997b; Dellas *et al.*, 1999; Kirchhoff *et al.*, 1999; Kirchhoff *et al.*, 2001). Analysis of preinvasive lesions has shown that CNI do occur, albeit at lower frequencies (Heselmeyer *et al.*, 1996; Kirchhoff *et al.*, 2001; Umayahara *et al.*, 2002). The presence of non-random aberrations in preinvasive lesions may point towards potentially important genes in the development of carcinoma. Heselmeyer and colleagues found gain in 3q to be present in 1/13 cases of CIN 3 (Heselmeyer *et al.*, 1996). As such this abnormality might define the transition to invasive disease, but others have found this aberration in CIN2, an earlier stage of preinvasive disease (Kirchhoff *et al.*, 2001).

Three recent studies have used CGH to study SCC of the vulva (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). The number of cases studied is small (36 in

total) but recurrent CNI were found in all studies. The pattern of CNI was similar to that found in SCC of the cervix. The most common losses affected 3p, 4p, 5q and 11q. In keeping with the cervical SCC, gain of 3q was the most frequent amplification seen. Allen and colleagues found this change to be limited to HPV-positive tumours and suggested 3q may harbour genes that act in synergy with HPV to cause anogenital carcinomas (Allen *et al.*, 2002). To date no CGH studies of vulval pre-malignancy have been performed.

In addition to understanding the critical steps in carcinogenesis, the study of CNI may provide useful prognostic information. In endometrioid adenocarcinoma of the uterine corpus the average number of CNI was significantly greater in those patients who died from their disease than for those patients who were disease free after treatment (Suehiro *et al.*, 2000). In addition, Suehiro and colleagues showed that the specific pattern of CNI could predict the likelihood of cervical involvement, lymph node spread and even death from the disease. More recently, Kiechle *et al.* showed a correlation between the pattern of CNI and the degree of differentiation for ovarian carcinoma (Kiechle *et al.*, 2001), itself a known prognostic variable.

1.3 The aims of this thesis

Vulval intraepithelial neoplasia is increasing in incidence, particularly amongst younger women. The rate of disease progression remains uncertain with estimates varying from 5-10% in treated patients to as high as 85% in untreated individuals. VIN shows a strong association with HR-HPV types that have already been linked with the malignant progression of other genital epithelial tumours. Current therapeutic options for affected individuals are unsatisfactory, often mutilating and carry disappointingly high recurrence rates. There is a pressing need to improve the understanding of the disease at a molecular level and to develop novel therapeutic options.

The research presented in this thesis aims to investigate viral and genetic factors that contribute to pathogenesis in VIN. It is hoped that this work will facilitate the identification of markers of disease progression, which might be used to identify women with a high or even inevitable risk of progression to invasive disease. In addition, a possible immunotherapy will be assessed in a phase II open-labelled clinical trial.

Within the confines of this project the following hypotheses will be tested:

- Within HPV positive disease the pattern of viral gene expression may influence disease progression
- In common with its cervical counterpart, VIN will exhibit non-random chromosomal alterations (both losses and gains).
- The degree and site of these abnormalities are crucial for containment of the lesion within the epithelium
- These abnormalities may differ between HPV positive and negative women
- Stimulating cell-mediated immunity against the most common HR-HPV genes may cause regression of clinical disease.

Chapter 2 Materials and Methods

2.1 Equipment and reagents

This sections lists equipment, reagents and suppliers used for this research.

2.1.1 General laboratory equipment

Specialised equipment will be discussed in the appropriate section of the methods.

Equipment	*Manufacturer*
Pipettes (2-1000µl)	Gilson
Pipette (large volume)	Integra Biosciences Ltd.
Bench top vortex	Chiltern Scientific
Bench top centrifuge	Heraeus
Centrifuge	Beckmann
Water baths	Grant
UV Spectrophotometer	Beckman Coulter
Hot block	Techne
Water purification – Elix10	Millipore
Water purification – Milli-Q	Millipore
PCR thermal cycler	Techne
PCR thermal cycler	Hybaid
Gel Imaging system/software	Flowgen
Laminar flow cabinet (class II)	Envair
$C0_2$ incubator	Heraeus
Dissecting microscope	Kyowa
Phase contrast microscope	Nikon
Microscope	Leitz
Microscope image capture system	Olympus

Table 2-1. General laboratory equipment.

2.1.2 Reagents and plasticware

General laboratory reagents were supplied by BDH laboratory supplies. Table 2-2 lists specific additional reagents, proprietary kits and plasticware used during the research project. Unless certified clean, plasticware was steam-autoclaved prior to use.

	Name	**Supplier**
Reagents	Amberlite IRN-150L	BDH Laboratory Supplies
	Anti-Digoxigenin-AP Fab	Roche Diagnostics Ltd.

Name	Supplier
fragments (sheep)	
Anti-digoxigenin-fluoroscein Fab fragments (mouse)	Roche Diagnostics Ltd.
Biotin 16-dutp	Roche Diagnostics Ltd.
Block	Roche Diagnostics Ltd.
Bovine Serum Albumin	Sigma-Aldrich
CGH Metaphase Target Slides	Abbott Diagnostic Division
dATP PCR grade	Roche Diagnostics Ltd.
Depex	BDH Laboratory Supplies
Denhardts solution	Sigma-Aldrich
Diaminobenzidine (DAB)	Sigma-Aldrich
Dig-RNA labeling mix	Roche Diagnostics Ltd.
Dig- RNA labelling kit	Roche Diagnostics Ltd.
Dig-11-dUTP	Roche Diagnostics Ltd.
1Kb+ DNA Ladder	Invitrogen Life Technologies
DNAZap	Ambion (Europe) Ltd.
DOP PCR master	Roche Diagnostics Ltd.
dTTP PCR grade	Roche Diagnostics Ltd.
Faramount aqueous mountant	Dako Ltd.
Ficoll-Paque PLUS	Amersham Biosciences Ltd
Fluorolink Cy3 streptavidin	Amersham Biosciences Ltd
Goat anti-mouse biotinylated antibody	Dako Ltd.
Goat Anti-Rabbit biotinylated antibody	Dako Ltd.
HotStar Taq. DNA polymerase	Qiagen Ltd.
Human Cot1-DNA	Invitrogen Life Technologies
Hybridisation Buffer	Sigma-Aldrich
Lymphoprep	Nycomed Pharma
B-mercaptoethanol	Sigma-Aldrich
NBT/BCIP Stock Solution	Roche Diagnostics Ltd
Normal Goat Serum	Dako Ltd.
NTP Mix (10mM each NTP)	Roche Diagnostics Ltd
OCT Compound	Agar Scientific Ltd
Optimised-200 Buffer Kit D	Invitrogen Life Technologies
Orthoboric Acid	Merck Ltd
Paraformaldehyde	Sigma-Aldrich
Polyoxyethylene ether W1	Sigma-Aldrich
Proteinase K	Sigma-Aldrich
Set of deoxynucleotides-PCR	Roche Diagnostics Ltd
Streptavidin-biotin antibody complex (SABC)	Dako Ltd.
Super Taq (HC)	HT Biotechnology Ltd.
N-Tris(hydoxymethyl)-3-aminopropanesulphonic acid (TAPs)	Sigma-Aldrich
Taq DNA Polymerase	Amersham Biosciences Ltd
Trizma base	Sigma-Aldrich
Tween-20 (Polyoxyethlenesorbitan	Sigma-Aldrich

	Name	Supplier
	monolaurate)	
	Vectashield (with DAPI) mountant	Vector Laboratories
	Water -DNase/RNase free	Sigma-Aldrich
Plasticware	0.65ml Multi-Ultra PCR tubes	Bioquote Ltd
	0.65-2ml reaction tubes	STARLAB (UK) Ltd.
	10-1000µl filter tips	STARLAB (UK) Ltd.
	ART 10 reach filter tips	Fisher Scientific
	1.8ml Cryotubes	Nalge (Europe) Ltd.
	Tissue Moulds	Nalge (Europe) Ltd.
	7.5ml S-Monovette tubes	Sarstedt Ltd.
Proprietary Kits	Qiaquick Gel extraction kit	Qiagen Ltd.
	Qiaquick PCR purification kit	Qiagen Ltd.
	Wizard Plus SV miniprep kit	Promega UK
	Plasmid midi-prep kit	Qiagen Ltd.
	DNA mini kit	Qiagen Ltd.
	Dig-RNA labelling kit	Roche Diagnostics Ltd.

Table 2-2. Reagents, plasticware and proprietary kits

2.1.3 Buffers and solutions

Buffers and solutions are listed in alphabetical order for ease of location. Unless otherwise stated the water used in preparing these solutions was purified by reverse osmosis and deionised using the Millipore Elix-10 system. Ultrapure water water for general PCR was obtained from the Millipore Milli-Q system. In addition to the above treatment this water was UV treated, ultrafiltered to remove particles >5000 Daltons and finally filtered through a 0.22µm membrane. For ease of reference, solutions and buffers are listed alphabetically and uses are specified (where appropriate).

Buffer 1
Use: RNA in situ hybridisation
Volume: make up to 500ml with water

Component	*Final concentration*	*Stock concentration*	*Volume of Stock (ml)*
Tris (pH 7.5)	0.1M	1M	50
NaCl	0.1M	5M	10
$MgCl_2$	2mM	1M	1
Triton X-100	0.05%	20%	1.25

Buffer 2
Use: RNA in situ hybridisation.
0.5% blocking reagent (Roche Diagnositics Ltd.) in buffer 1.

Buffer 3
Use: RNA in situ hybridisation.
Volume: make up to 500ml with water

Component	*Final concentration*	*Stock concentration*	*Volume of Stock (ml)*
Tris (pH 9.5)	0.1M	1M	50
NaCl	0.1M	5M	10
MgCl2	50mM	1M	25

DNA loading buffer
Use: loading DNA for gel electrophoresis
Volume: make up to 60ml with water

Component	*Amount*
EDTA pH 8.0	3ml 0.5M stock
Ficoll 400	15g
Bromophenol blue	0.05g
Xylene cyanol	0.05g

Bromophenol blue marker runs at ~400bp; xylene cyanol at ~1500 bp

Hybridisation buffer – CGH
Use: resuspending DNA probe prior to CGH
Volume: make up to 50 ml with water

Component	*Final concentration*	*Stock concentration*	*Volume of Stock (ml)*
Formamide	25%	50%	25
Dextran Sulphate	10%	50%	10
SSC	2x	20x	5
Sodium Phosphate buffer*	40mM	0.5M	4
Denhardts Solution	1x	50x	1

*0.5M sodium phosphate buffer, pH 7: 288mM Na_2HPO_4 + NaH_2PO_4
Combine reagents thoroughly and mix. Filter sterilise, aliquot and store frozen at –20°C.

1kb+ ladder (quantitative)
Use: quantifying agarose gel electrophoresis products.
Volume: 1.2ml

Component	*Amount (µl)*
1kb+ ladder	75
DNA loading buffer	200
Water	925

When 10µl is used as reference ladder, the 1650 base marker band is 50ng.

Lysis Buffer
Use: DNA extraction
Volume: 500µl (or multiples thereof)

Component	*Final concentration (mM)*	*Stock concentration (M)*	*Volume of Stock (µl)*
Trizma base (pH 7.5)	10	1	50
EDTA (pH7.5)	10	1	50
Water	n/a	n/a	400

Make up fresh for each extraction. Keep on ice.

Phosphate buffered saline (PBS) - 10x stock

Volume: 1000ml

Component	*Final concentration*	*Amount (g)*
NaCl	1.37M	81.00
$Na_2HP0_4.12H_20$	0.1M	19.80
$Na_2HP0_4.H_20$	22.4mM	1.57

Add water to 800ml and pH to 7.4 with HCl. Make up to 1L with water and autoclave.

Polyoxyethylene ether W-1 (W-1 detergent)

Use: Degenerate oligonucleotide primed PCR.

Volume: 100ml

Dissolve 1g of W-1 in 80ml water, warming solution to 65°C to facilitate process. Make up to 100ml with water, filter-sterilise (22µm), aliquot and store at -20°C.

Proteinase K buffer

Use: RNA in situ hybridisation.

Volume: 500ml

Component	*Final concentration*	*Stock concentration*	*Volume of Stock (ml)*
Tris (pH 7.4)	20mM	1M	10
$CaCl_2$	2mM	0.1M	10

3M Sodium Acetate pH 5.2

Use: Nucleic acid precipitation

Volume: 100ml

Dissolve 40.82g sodium acetate tri-hydrate in 50ml water. Adjust pH to 5.2 with glacial acetic acid. Increase volume to 100ml with water. Autoclave prior to use.

Salt sodium Citrate (SSC) – 20x stock

Use: In situ hybridisation (including comparative genomic hybridisation)

Volume: 1000ml

Component	*Amount (g)*
Sodium chloride	175.3
Tri-sodium citrate	88.2

Dissolve in 800ml water. Adjust pH (pH 7.0 with NaOH (5M); pH 5.2 with concentrated HCl) and make up to 1000ml with water.

SST – 4x stock
Use: Comparative genomic hybridisation
Volume: 500ml

Component	*Volume*
20x SSC-neutral	100ml
Water	400ml
Tween -20	250µl

N-Tris (hydroxymethyl)-3-aminopropanesulphonic acid (TAPS) buffer
Use: Degenerate oligonucleotide primed (DOP) PCR
Volume: 40ml

Component	*Final concentration*	*Amount*
TAPs	250mM	2.43g
KCl	500mM	1.49g
$MgCl_2$ (1M)	20mM	80µl
β-mercaptoethanol	28.8µM	280µl

Make up solution in 30ml water before adjusting pH to 9.3 with NaOH (5M). Adjust volume to 40ml with water. Filter-sterilise (22µm), aliquot and store at -20°C.

Tris-Borate-EDTA (TBE) buffer - 10x stock
Use: Running buffer for gel electrophoresis
Volume: make up to 2000ml with water

Component	*Final concentration (mM)*	*Amount (g)*
Trizma base	89	216
Boric acid	89	110
Di-sodium EDTA	2.6	19

Dissolve in 1800ml water before adjusting pH to 8.3 with concentrated HCl.

Tris-EDTA (TE) buffer (10:1mM) – 10x stock
Use: DNA extraction
Volume: make up to 1000ml with water

Component	*Final concentration /mM*	*Amount /g*
Trizma base	100	12.1
EDTA	10	3.72

Dissolve in 800ml water. Adjust pH to 8.0 with concentrated HCl.

2.1.4 Tissue Culture

	Name	*Supplier*
General Media	Glasgow's Modified Eagle's Medium (GMEM)	Sigma-Aldrich
	Dubeccio's Modified Eagle's Medium (DMEM)	Sigma-Aldrich
	RPMI - 1640	Sigma-Aldrich
Additive	Fetal calf serum (10x)	PAA Laboratories Ltd.

	Name	*Supplier*
Additive cont)	Glutamine (100x)	Invitrogen life technologies
	Penicillin/ Streptomycin (10,000u/ml and 10,000µg/ml)	Invitrogen life technologies
	Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
	Trypsin/EDTA (0.5%/5.3mM)	Invitrogen life technologies
Plasticware	50ml polypropylene tubes	Falcon
	1.8ml polypropylene cryotubes	Nalge (Europe) Ltd.
	Sterile Flat culture flask (25, 75 and 125 cm^2)	Falcon
	Sterile plastic pipettes (10 and 25ml)	LPA Italiana SPA

Table 2-3. Tissue culture materials

2.1.4.1 Additional media

The following media were prepared as required in the laboratory:

Luria Broth (LB)
Volume: 1000ml

Component	*Amount (g)*
Tryptone	10
Yeast extract	5
NaCl	10

Dissolve in 800ml and adjust to pH 7.5 with NaOH (5M). Make up to 1000ml and autoclave.

Tryptone Yeast culture medium - 2x (2TY)
Volume: 1000ml

Component	*Amount (g)*
Tryptone	16
Yeast extract	10
NaCl	5

Dissolve in 800ml. Make up to 1000ml and autoclave.

SOB
Volume: 1000ml

Component	*Final concentration*	*Amount (g)*
Bactotryptone	2%	20
Yeast extract	0.5%	2
NaCl	10mM	0.584
KCl	2.5mM	0.186
$MgCl_2$	10mM	See below
$MgSO_4$	10mM	See below

Prepare without $MgCl_2$ and $MgSO_4$ and autoclave. To 100ml add 1ml of 1M $MgCl_2$ and 1ml 1M $MgSO_4$ just before use (**SOC**).

TFB

Volume: variable

Reagent	*Concentration (mM)*
K-MES	10
KCL	100
$MnCl_2.4H_2O$	45
$CaCl_2.6H_2O$	10
Hexamine $CoCl_3$	3

K-MES is 1mM morpholino ethanesulfonic acid (MES) adjusted to pH 6.3 with KOH and filter sterilised. TFB stored in 10ml aliquots at -20°C.

2.2 METHODS

2.2.1 DNA sources and extraction

DNA was extracted from both clinical specimens and tissue culture. Ethical approval for the project was given by Cambridge Local Research Ethics Committee (LREC 98/227). Data collected concerning the clinical specimens was kept in accordance with hospital guidelines on data protection and the research database was registered under the 1998 Data Protection Act.

2.2.1.1 Handling of clinical specimens

2.2.1.1.1 Fresh tissue biopsies

Fresh tissue specimens were collected from patients in the Colposcopy and Vulval clinics as well as the Gynaecological Oncology theatres at Addenbrookes Hospital Cambridge. Specimens for HPV typing were place in cryotubes and immediately snap frozen in liquid nitrogen before being stored at -70°C.

Where samples were required for CGH the biopsy was split using a sterile blade and half the biopsy was mounted on edge in a tissue mould using OCT compound before storage at -70°C. Subsequent manipulation of tissue samples was carried out in the category 2 area of the Division of Virology, University of Cambridge.

2.2.1.1.2 Blood

Blood specimens were taken for immunological studies and control specimens. Sterile technique was used in the collection of samples. Samples were either processed or transferred to the collaborating laboratory at Xenova Research Ltd. without delay.

2.2.1.1.3 Isolation of mononuclear cells from whole blood

Approximately 15ml of blood from the patient was transferred to a 50ml Falcon tube and mixed thoroughly with an equal volume of 0.9% sodium chloride by gentle inversion of the tube. A fresh Falcon tube was prepared containing 15ml of Lymphoprep. The blood/saline mixture was slowly added to the Lymphoprep so as to layer the blood/saline mix on top of the Lymphoprep. Care was taken to prevent mixing of the two layers. The layered solution was then centrifuged at 2000rpm (~800g) using a 'swing-out' rotor. The Falcon tube was then removed taking care not to disturb the 4 layers that will have formed. The mononuclear cells are to be found in the second layer and can be carefully drawn off with a pipette (layer order top→bottom is plasma, mononuclear cells, lymphoprep, neutrophils and red cells). The mononuclear aspirate was then transferred to a clean tube and to which ~3 volumes of 0.9% sodium chloride solution was added. The tube was inverted to aid mixing of the liquids before centrifuging gently at 1000rpm (~100g) for a further 10 minutes. The supernatant was discarded and the mononuclear cell pellet resuspended in 2ml of 0.9% sodium chloride. The cell concentration was then estimated using a cytometer and an appropriate volume used for DNA extraction as detailed below.

2.2.1.2 Tissue culture

HPV-positive and HPV-negative cell lines were used to provide control DNA for PCR experiments.

2.2.1.2.1 Cell lines

The following table details the cell lines used during this research. Frozen stocks were kindly provided by Dr. Jane Sterling, Division of Virology, University of Cambridge.

Cell Line	*Origin*	*HPV status*	*Reference*
HaCaTs	Immortalised aneuploid human keratinocytes	HPV negative	(Boukamp *et al.*, 1988)
SiHa	Human epidermoid cervical carcinoma cells	1-2 copies of integrated HPV 16 per Cell	(Friedl *et al.*, 1970)
CaSki	Human epidermoid cervical carcinoma cells	~600 copies of HPV 16 per cell (integrated as concatamers)	(Pattillo *et al.*, 1977)

Table 2-4. Cells lines used for control DNA

2.2.1.2.2 Cell culture technique

Cell culture preparation was carried out under sterile conditions in a Class II Laminar flow hood. Clean laboratory coats and gloves were available for this purpose and all equipment was wiped with 70% Ethanol prior to use. Cell line specific media are detailed in the following table.

Cell line	*Media*	*Additives per 500ml media*
HaCaTs	GMEM	10% FCS; 2mM Glutamine; 100u Penicillin; 100µg streptomycin.
SiHa	DMEM	10% FCS; 2mM Glutamine; 100u Penicillin; 100µg streptomycin.
CaSki	RPMI-1640	10% FCS; 2mM Glutamine; 100u Penicillin; 100µg streptomycin.

Table 2-5. Cell line-specific media

2.2.1.2.3 Resuscitation of stored cell stocks

Cryotubes containing 1.5ml aliquots of cells (~2x10^{6}cells per ml) frozen in the appropriate medium with 10% DMSO and stored in liquid nitrogen were thawed at 37°C before being transferred to 5-10ml of appropriate pre-warmed culture medium in a 50ml Falcon tube. The cells were separated by centrifuging at 1500rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in the appropriate pre-warmed culture medium. The volume of medium used was dependent on the size of tissue culture flask to be used (see

table). The cell pellet was initially resuspended in one third of the appropriate volume of media, the rest being added directly to the tissue culture flask. The resuspended cells were then transferred gently to media in the flask. The flasks closed with the lids in the 'vent' position and placed at 37°C in a 5% CO_2 incubator. Cell cultures were reviewed at 24 hours and the medium exchanged for fresh pre-warmed medium. Cells were passed at ~90% confluence.

Flask size (cm^2)	Suggested volume of media (ml)	Maximum volume (ml)
25	7	8
75	15	20
125	30	40

Table 2-6. Suggested volumes of media for varying flask sizes

2.2.1.2.4 Passing of confluent cells

The old culture medium was poured from the flask and the 'monolayer' culture washed twice with 20ml PBS. 5ml Trypsin/EDTA mix was added to 15ml PBS (total volume 20ml) and the solution added to the culture flask. The flasks were then incubated for ~2minutes at 37°C until the cells rounded-up and started to lift. Detachment of the cells was aided by tapping the flask. The cell solution was then added to an equal volume (20ml) of fresh culture medium in a 50ml tube to quench the trypsin and the cells pelleted by centrifuging at 1,500rpm for 2-4 minutes. The supernatant was poured away and the cell pellet gently resuspended in 10ml of fresh medium. The cells were then split (1/5-1/10) to a new culture flask into which the appropriate volume of culture medium had already been added (Table 2-6). Culture then proceeded as above.

2.2.1.2.5 Preparing frozen stocks

A freezing mix of 20% DMSO and media was prepared and kept cold. The cells were harvested as above and resuspended in 10 ml and the cell concentration estimated using a cytometer. An appropriate volume of the suspension was then pelleted using a centrifuge to yield approximately $2x10^6$ cells. This cell pellet was then resuspended in 1-1.5ml DMSO/Media mix and transferred to a cryotube. The cryotube of cells was then put into an insulated box and placed at

-70°C overnight. The next day the frozen cell stock was transferred to the liquid nitrogen storage container.

2.2.1.3 DNA extraction

Samples for HPV typing were extracted in a separate area to the usual workspace to avoid possible contamination with HPV DNA.

2.2.1.3.1 DNA extraction from tissue biopsies

Human tissue biopsies were handled in the category 2 area of the Division of Virology, University of Cambridge. Biopsies were morcellated in sterile Petri dishes and the pieces placed into a 1.5ml tube containing 500µl of extraction buffer (comprising 465µl cold lysis buffer, 10µl proteinase K (10µg/µl) and 25µl SDS (10%)). The tube was agitated for 18hours (overnight) at ~37°C. After inspection to ensure appropriate digestion the proteinase K was heat inactivated by heating to 95°C for 5-10 minutes. After a short period of centrifugation to remove drops from the lid of the tube, the solution was cleaned using the phenol/chloroform method. Briefly, an equal volume of phenol was added to the tube, before pulse-vortexing to mix the liquids. The mixture was then centrifuged at 13,000rpm for 1 minute to allow separation of aqueous and phenolic phases. The phenol was removed from under the aqueous phase using a 'drawn out' sterile glass pipette. An equal volume of chloroform was then added and the mixture/centrifugation/removal process repeated. In order to ensure optimal purity of the DNA samples this whole process was repeated a second time. Finally the aqueous phase was transferred to a fresh 2ml tube. DNA was precipitated from solution by adding 1/10 volume of 3M sodium acetate and 2 ½ times the volume of 100% ethanol. After thorough mixing the tube was placed at -70°C for 30 minutes, or at -20°C overnight. The precipitated DNA was centrifuged at 13,000rpm for 30minutes before washing the pellet in 70% ethanol. After further centrifuging (13,000rpm, 20 minutes) the excess ethanol was carefully decanted and the DNA pellet dried in a vacuum desiccator before re-suspending in 150-200µl of ultrapure water.

2.2.1.3.2 Extraction of DNA from cells

In contrast to the tissue specimens, large quantities of cells (from culture or blood) were available for DNA extraction. To maximise sample purity DNA was extracted from these samples using a proprietary kit (QIAamp DNA mini kit, QIAGEN). This method involves proteinase K digestion and DNA isolation using spin columns and a microcentrifuge. The lysate buffer provides optimal pH conditions for binding to the spin column membrane. The membrane can then be washed with two different wash buffers to remove residual contaminants. After an additional centrifugation step to ensure all the wash buffer had been removed, the DNA was eluted from the column membrane using water.

2.2.1.3.3 Extraction of DNA from microdissected frozen sections

Frozen sections were cut in the Department of Histopathology, Addenbrooke's hospital, Cambridge. Sections were cut at a thickness of 7-10μm onto uncoated slides, taking care not to thaw the sections onto the slides. The microtome blade was changed or cleaned thoroughly (xylene/HCL) between blocks to prevent cross-contamination. Slides were stored at -70°C until required. A reference section from either end of the run was stained with haematoxylin and eosin (H/E) for histopathological review. Six to 10 sequential sections were then thawed into 70% ethanol to fix the slides, stained with H/E and prepared for microdissection as detailed below:

Solution	*Time*
70% Ethanol	30s
H_20	30s
Haematoxylin	30s
H_20	30s
70% EtOH	30s
90% EtOH	30s
Eosin	30s
70% EtOH	30s
90% EtOH	30s
100% EtOH	30s
Xylene	5 mins

Table 2-7. Haematoxylin and Eosin staining protocol for frozen sections

After staining and dehydration the slides were allowed to air dry. The target area of epithelium was identified for each case. The cells were then dissected under stereoscopic visualisation (10-15x magnification) using a sterile 25G needle mounted on a 5ml sterile syringe. Only moderate pressure was required and the dissected tissue tended to stick to the needle with static charge. The cells were then transferred into 60µl extraction buffer (comprising 1mg/ml proteinase K, 1% Tween 20 and TE buffer pH8.0) in a thin walled 0.5ml tube. The extraction mixture for one set of extractions was prepared as a batch mixture and aliquoted, retaining 1-2 aliquots for use as controls in subsequent PCR reactions. The tube was placed in a hotblock/thermal cycler at 38°C for 18 hours. The temperature was then raised to 95°C for 10 minutes to inactivate proteinase K and the resulting solution used neat in subsequent PCR reactions.

2.2.1.3.4 Histopathological review

All cases were reviewed by two independent consultant gynaecological pathologists (Dr N. Coleman, Hutchinson MRC Cancer Cell Unit and Dr R. Moseley, Addenbrooke's NHS Trust). Where discrepancies in classification were found both pathologists reviewed the sample and reached a consensus decision.

2.2.1.3.5 Assessing DNA concentration

Where sufficient volumes of DNA solution were available, an estimate of DNA concentration and purity was made using the sample absorbance at 260nm and 280nm (A_{260} and A_{280}). DNA at a known dilution (in 1ml total volume with water) was placed in a quartz cuvette and the A_{260} measured in a spectrophotometer after calibration with an appropriate water control. The concentration of DNA can be estimated by the following equation

Concentration (µg/ml) = A_{260} x OD constant for DS-DNA x dilution factor

where A_{260} is the sample absorbance at 260nm (OD) and the OD constant for DS DNA is 50µg/ml. Purity of the sample was assessed by calculating the ratio of A_{260}:A_{280} which should be ≥1.6 and ideally ≥1.8.

For samples where there was insufficient total volume to allow for repeat spectrophotometry to calculate the DNA concentration e.g. clinical PCR products, a known volume of sample was run out on a 1% agarose gel with two references ladders of known concentration. The 1650bp band of 1 Kb plus DNA ladder (Invitrogen Life Technologies) contains ~8% of the total mass applied to the gel. The ladder was therefore prepared in such a fashion that 10µl of ladder would have a 1650bp band equivalent to 50ng. An image of the gel was captured with 10µl and 20µl of ladder as reference standards (50 and 100ng). The image capture software (Flowgen) was used to define test and control bands, as well as gel background signal. The concentrations of the control bands were then specified to allow a standard curve to be constructed. DNA concentrations for the unknown products could then be estimated by measuring the optical densitiy of the unknown bands compared to those of the known controls.

2.2.2 Polymerase chain reaction

This section details the PCR methodology used during this thesis. Degenerate oligonucleotide primed PCR will be discussed in a later section.

2.2.2.1 PCR – general considerations

The polymerase chain reaction is an exquisitely sensitive primer extension reaction for detecting and amplifying nucleic acids *in vitro* (Saiki *et al.*, 1988). Using thermostable polymerases with thermal cycling machines ~10^5 fold amplification is achievable.

2.2.2.1.1 Thermal cyclers

Two thermal cyclers were used during this work and have been listed earlier. The first (Progene, Techne) operated by the 'block control' system, where the temperature of the block was varied and a predetermined algorithm used to estimate the reaction mixture temperature within the thin walled PCR reaction tubes. The second machine (PCR express, Hybaid) used a thermistor sensor to regulate tube temperature. This thermistor was placed into a PCR tube containing mineral oil, which was positioned in the thermal block. The

thermistor attempts to provide an accurate measurement of the actual temperature within the reaction tubes. The samples are then held at the precise temperature required for the specified time. This avoids the lag associated with block control machines where the sample temperature lags behind the programmed block temperature. The precise nature of the timing/temperature control allows a reduction in incubation times for each step. This machine also has a gradient block, which allows a temperature gradient to be programmed across the block, thereby enabling a range of annealing temperatures to be investigated during a single PCR run.

2.2.2.1.2 Contamination prevention

The sensitivity of PCR is in itself a potential problem. Stringent methods were therefore adopted to prevent contamination. Reagents for PCR were stored at -20°C in a separate freezer area from DNA and all PCR buffers and solutions were aliquoted on receipt to reduce the risk of contamination being introduced to the stocks. The thermostable polymerases used were solely for the use of the investigator and were not part of the general laboratory stocks. The risk of contamination was further reduced by using dedicated, separate areas for the preparation of PCR solutions. Briefly, the reagents for a PCR reaction were made up into a 'master-mix' in a dedicated area within the Division of Virology. The 'master-mix' contained the common reagents required for all the PCRs to be set up. Aliquots of the mix can then be dispensed into each reaction tube. This reduces the number of pipetting steps and thus the potential for cross contamination between tube. Dedicated pipettes with filter-tips were used for this process. The equipment was kept in a sealed container and the bench space and container exterior were cleaned with an anti-DNA preparation (DNA-Zap, Ambion Ltd.) both prior to and following use. This product is a proprietary DNA/RNA degradation solution that is capable of degrading any nucleic acid down to free nucleotides. To avoid potential contamination by human DNA from the investigator a clean laboratory coat and gloves were worn. Target DNA was added to the reaction tubes in another area of the laboratory using a different set of filter tips and pipettes. No other investigators were using HPV

DNA in this area. Finally, the products of the PCR reaction were manipulated in a third bay.

2.2.2.1.3 Controls

All PCR reactions were run with appropriate negative and positive control reactions. For β-globin PCRs these included reactions with no template, the extraction buffer and placental DNA. For HPV detection this includes reactions with no template (water/extraction buffer), HPV negative DNA and HPV positive DNA at a known copy number. For DOP-PCR, the extraction mix and filter PCR grade water were used as negative controls. Amplification of a known amount of the control DNA (~300ng) served as a positive control. Results from a PCR experiment were only used if all controls gave appropriate results.

2.2.2.1.4 Hot-start PCR

The degenerate-primers used in the HPV detection PCR systems are prone to non-specific amplification and the formation of primer-dimers. To reduce these deleterious effects a hot-start enzyme was used (HotStarTaq, Qiagen). This recombinant Taq DNA polymerase requires a prolonged period of heat activation (15 minutes at 95°C) before it exhibits any polymerase activity. As such the enzyme can be added to the 'master-mix' without the worry of non-specific product formation. All reaction components can be prepared at room temperature without the need to keep samples on ice. The prolonged initial heating step also ensures thorough denaturation of the target DNA.

2.2.2.2 Preparation of control DNA from HaCaT, CaSki and SiHa cell lines

HaCaT and CaSki or SiHa cell lines were used as a source of HPV-negative or -positive control DNA respectively (Table 2-4). The cell concentrations in cell suspensions were estimated using a haemocytometer. A volume of suspension equivalent to 1x 10^6 cells was then used as a DNA source. DNA was extracted as using a proprietary kit (QIAamp DNA mini kit, QIAGEN) in the fashion described previously (section 2.2.1.3.2). The DNA was then eluted into a known volume (600μl). If the process is assumed to be completely efficient, the

concentration of copies of HPV 16 can be estimated. Dilution of the DNA extract provides the opportunity to establish the sensitivity threshold for the various PCR systems used. HaCaT DNA is HPV-negative and provides an opportunity to investigate non-specific amplification in the PCR systems. The use of these controls will be further described in Chapter 3.

2.2.2.3 Primer sequences

The sequences and characteristics of the primers used during PCRs performed as part of this research are listed in the following table:

Use	*PCR*	*Primer*	*Bases*	*Sequence 5' – 3'*	*Tm (°C)*	*Product size (bp)*
HPV typing	β-globin	Forward	20	GAAGAGCCAAGGACAGGTAC	54	268
		Reverse	20	CAACTTCATCCACGTTCACC	52	
	GP5+/6+	Forward	23	TTTGTTACTGTGGTAGATACTAC	50	~150
		Reverse	25	GAAAAATAAACTGTAAATCATATTC	46	
	MY09/11	Forward	20	CGTCCMARRGGAWACTGATC	50-56	~450
		Reverse	20	GCMCAGGGWCATAAYAATGG	50-54	
			Degenerate base code **W** = A or T; **Y** =C or T; **R** = A or G and **M** = A or C.			
Riboprobe synthesis	HPV16 E1	Forward	20	GCGGATCCGGGATGTAATGG	56	382
		Reverse	26	GCGAATTCAAGCTTATACCCGCTGTG	60	
	HPV16 E2	Forward	21	GCGGATCCTACAAGGCCAGAG	58	455
		Reverse	26	GCGAATTCAAGCTTGCATGAACTTCC	58	
	HPV16 E4	Forward	20	GCGGATCCCGAAGTATCCTC	56	278
		Reverse	26	GCGAATTCAAGCTTCTATGGGTGTAG	58	
	HPV16 E6	Forward	23	GCGGATCCATGCACCAAAAGAGA	57	497
		Reverse	28	GCGAATTCAAGCTTTACAGCTGGGTTTC	60	
	HPV16 E7	Forward	24	GCGGATCCATGCATGGAGATACAC	59	317
		Reverse	27	CGAATTCAAGCTTTATGGTTTCTGAGA	55	
	HPV16 L1	Forward	20	GCGGATCCGTAAGCACGGAT	56	367
		Reverse	26	GCGAATTCAAGCTTCTGCATAAGCAC	58	
	HPV 16 L2	Forward	22	GCGGATCCATGCGACACAAACG	59	385
		Reverse	26	GCGAATTCAAGCTTATGTTGGTGCAC	58	

Use	*PCR*	*Primer*	*Bases*	*Sequence 5' – 3'*	*Tm (°C)*	*Product size (bp)*
Integration PCR	HPV16 E2	Forward	19	AGGACGAGGACAAGGAAAA	49	1139
		Reverse	20	GGATGCAGTATCAAGATT TG	48	

NB. Tm = melting temperature – calculated by 'basic' method 4x(G+C)+2x (A+T). Actual temperature quoted by supplier will be salt-adjusted and higher.

Table 2-8. Primer sequences, Tm and amplicon sizes for primer pairs

2.2.2.4 PCR reaction mixtures

Reagent	*Stock concentration*	*Reaction concentration*	*Volume /µl*
DNA template	n/a	n/a	2
Buffer	X10	X1	5
NTP	2 mM	0.2 mM	5
(MgCl2) **	25 mM	2.0/2.5 mM	(1/2)
Forward primer	10 µM	0.4 µM	2
Reverse primer	10 µM	0.4 µM	2
Taq polymerase	5 U/µl	0.05 U/µl	0.5
Water	n/a	n/a	33.5 (32.5/31.5)
		TOTAL	**50µl**

Table 2-9. Standard 50µl PCR reaction mixture.

** PCR buffer contains $MgCl_2$ at 15mM. Volume specified corresponds to additional volume of $MgCl_2$ added for MY09/11 and Integration PCRs respectively, to obtain final concentration as specified. Water volume adjusted accordingly.

A standard PCR reaction mixture was used for β-globin, riboprobe synthesis and degenerate HPV-typing reactions. Additional magnesium chloride was used to improve amplification in the HPV 16E2/16E7 integration PCRs. The standard reaction volume used was 50µl, but the reaction was scaled up as required to produce more product e.g. for sequencing. All reaction mixtures were made up as a 'master mix', prior to aliquoting into each reaction tube . DNA template was then added to each tube individually. To reduce the chance of calculation errors, the templates for each PCR were entered into an Excel spreadsheet and macros used to allow the rapid and accurate calculation of the volumes of reagent required.

2.2.2.5 PCR protocols

The following tables describe the PCR protocols used for β-globin, degenerate and HPV type-specific PCR. After an initial denaturing step at 95°C for 5 or 15 minutes (dependent on whether a 'standard' or 'hotstart' Taq was used), the subsequent denaturing steps for each cycle were reduced to 94°C for 30 seconds each cycle to avoid unnecessary adverse effects on the efficacy of the Taq-polymerase, which has a half life of about 30mins at 95°C. Following the terminal elongation step, samples were held at 4°C prior to storage at -20°C or assessment by gel electrophoresis.

Primer set	*Step*	*Temperature (°C)*	*Time (min)*	*cycles*
β-globin	Initial denaturing	95	5	1
	Amplification	94	0.5	
		56	1	35
		72	1	
	Terminal elongation	72	7	1
MY09/11	Initial denaturing	95	15	1
	Amplification	94	0.5	
		55	1	35
		72	1	
	Terminal elongation	72	10	1
GP5+/6+	Initial denaturing	95	15	1
	Amplification	94	0.5	
		50	2	40
		72	1.5	
	Terminal elongation	72	4	1
Riboprobe synthesis	Initial denaturing	95	5	1
	Amplification	94	0.5	
		56	1	35
		72	1	
	Terminal elongation	72	7	1

Primer set	*Step*	*Temperature (°C)*	*Time (min)*	*cycles*
16E2 Integration	Initial denaturing	95	15	1
	Amplificaton	94	0.5	
		59	1	38
		72	2	
	Terminal elongation	72	7	1
16E7 Integration	Initial denaturing	95	5	1
	Amplificaton	94	0.5	
		56	1	38
		72	2	
	Terminal elongation	72	7	1

Table 2-10. PCR protocols for various primer sets used. Following PCR all samples held at 4°C.

2.2.2.5.1 Nested-PCR for HPV typing

When the MY09/11 and GP5+/6+ primer systems were used in a nested fashion the PCR mix and thermal cycling programs detailed above were employed with the following exceptions:

1. 0.5 µl of MY09/11 PCR product was used for the template in the nested GP5+/6+ PCR.
2. The GP5+/6+ amplification step was reduced to 30 cycles from 40 cycles.

2.2.2.6 HPV typing

The GP5+/6+ primer system (de Roda Husman *et al.*, 1995) was used to amplify genital HPV types from clinical DNA. Ten microlitres of PCR product was run-out by electrophoresis on a 1.5% agarose gel, stained with ~0.5µg/ml ethidium bromide and viewed using UV transillumination. Gel images were recorded using a gel imager (Flogen) and stored in TIFF format files for reproduction. All reactions were repeated at least once to ensure the results were reproducible. The positive PCR products were purified using a proprietary kit (Qiaquick PCR purification kit, Qiagen Ltd.). This system relies on salt and pH-dependent

adsorption of DNA to a membrane within a spin column. The membrane is then washed to remove unwanted primers and contaminants before the DNA is eluted using low-salt solutions or water. Where only small amounts of product resulted from the degenerate PCR amplification, the reaction volume was scaled-up and performed several times. The PCR products were pooled, adsorbed onto a single spin column and eluted into a low volume e.g. 30µl to provide a suitable concentration of PCR product for sequencing. Approximately 20-30ng of GP5+/6+ PCR product was sent for sequencing at the DNA sequencing facility of the Department of Biochemistry, University of Cambridge. One of the primer pairs was used as the sequencing primer and the sequence determined by automated fluorescent dye-terminator cycle sequencing (Sanger *et al.*, 1977). The resulting sequence was viewed using Chromas software (Technelysium Pty Ltd, Australia). Obvious errors in the automated base allocation were corrected manually. The corrected sequence was then exported in FASTA format and compared using a modified BLAST search (Altschul *et al.*, 1997) against the known human papillomavirus sequences held in the Los Alamos sequence library (http://hpv-web.lanl.gov/stdgen/virus/hpv/). HPV type was allocated on the basis of > 90% homology for a continuous sequence of over 100 bases.

During the latter part of this work, the MY09/11 primer system (Manos *et al.*, 1989) was used to investigate those specimens found to be HPV-negative by GP5+/6+ PCR. If a clear product was visible on agarose gel electrophoresis then this was purified and sequenced in a similar fashion to the GP5+/6+ PCR product (see above), only using one of the MY09/11 primers as the sequencing primer. Equivocal or negative MY09/11 products were further subject to a nested PCR using the GP5+/6+ primers as the nested primers. Samples found to be positive on nested PCR were typed by purification and sequencing of the GP5+/6+ nested product in the manner described previously.

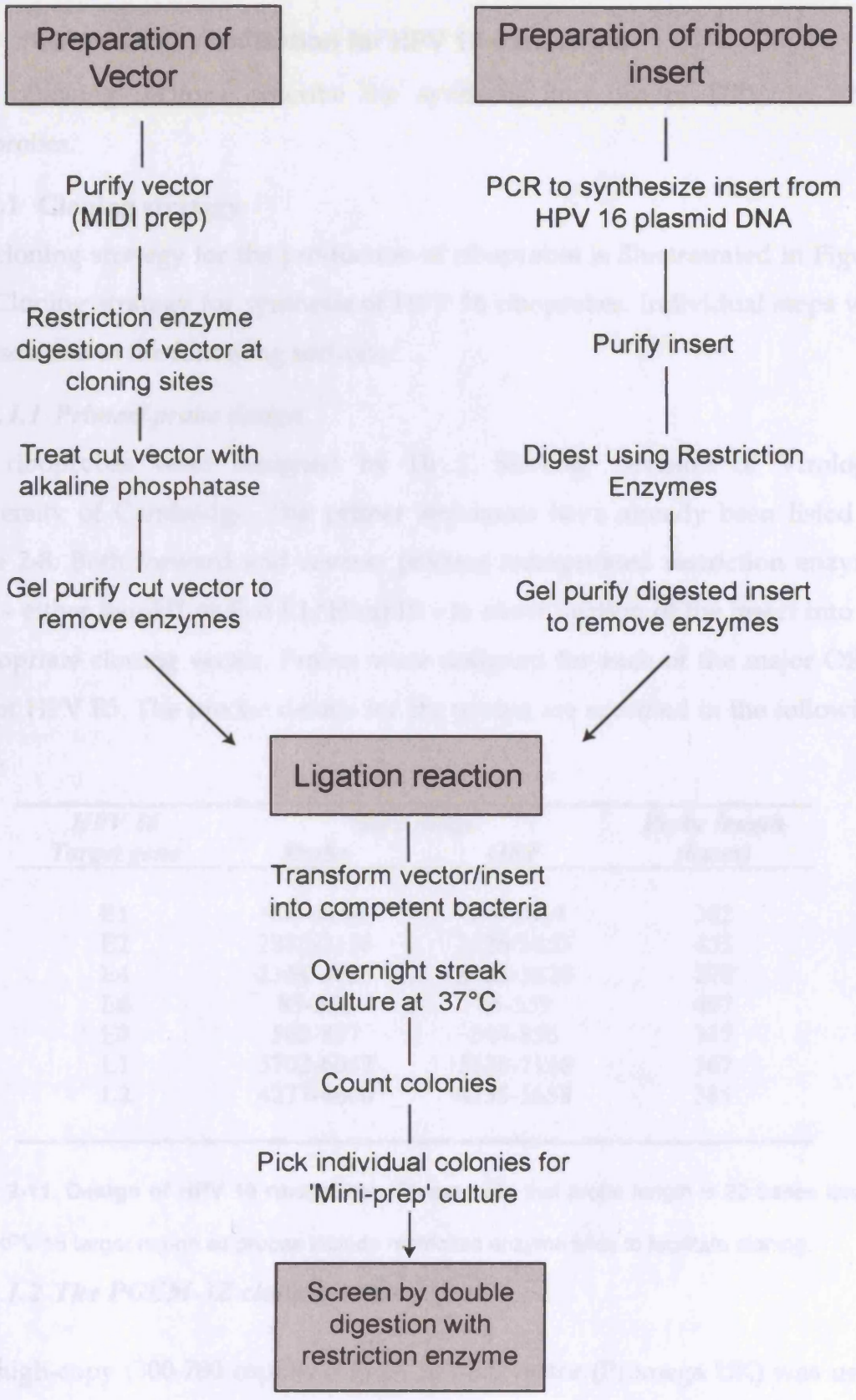


Figure 2-1. Cloning strategy for synthesis of HPV 16 riboprobes

2.2.3 RNA *in situ* hybridisation for HPV 16 transcripts

The following sections describe the synthesis and use of HPV 16 RNA riboprobes.

2.2.3.1 Cloning strategy

The cloning strategy for the production of riboprobes is illustratrated in Figure 2-1. Cloning strategy for synthesis of HPV 16 riboprobes. Individual steps will be discussed in the following sections.

2.2.3.1.1 Primer/probe design

The riboprobes were designed by Dr J. Sterling, Division of Virology, University of Cambridge. The primer sequences have already been listed in Table 2-8. Both forward and reverse primers incorporated restriction enzyme sites - either BamH1 or Eco R1/Hind III - to allow ligation of the insert into an appropriate cloning vector. Probes were designed for each of the major ORFs except HPV E5. The precise details for the probes are specified in the following table.

HPV 16 Target gene	*Base range*		*Probe length (bases)*
	Probe	*ORF*	
E1	906-1266	859-2814	382
E2	2885-3318	2726-3853	455
E4	3364-3620	3333-3620	278
E6	83-558	65-559	497
E7	562-857	544-858	317
L1	5702-6047	5528-7156	367
L2	4237-4600	4135-5658	385

Table 2-11. Design of HPV 16 riboprobes. Please note that probe length is 22 bases longer than HPV 16 target region as probes include restriction enzyme sites to facilitate cloning.

2.2.3.1.2 The PGEM-3Z cloning vector

The high-copy (300-700 copies/cell) pGEM-3Z vector (Promega UK) was used as a cloning vector. The vector has a multiple cloning region, which contains restriction enzyme sites for the three restriction enzymes encorporated into the RNA probes (BamH1, EcoR1 and HindIII). The vector has both the SP6 and T7

RNA polymerase sites flanking the multiple cloning region (Figure 2-2) to allow for the highly specific synthesis of RNA *in vitro*.

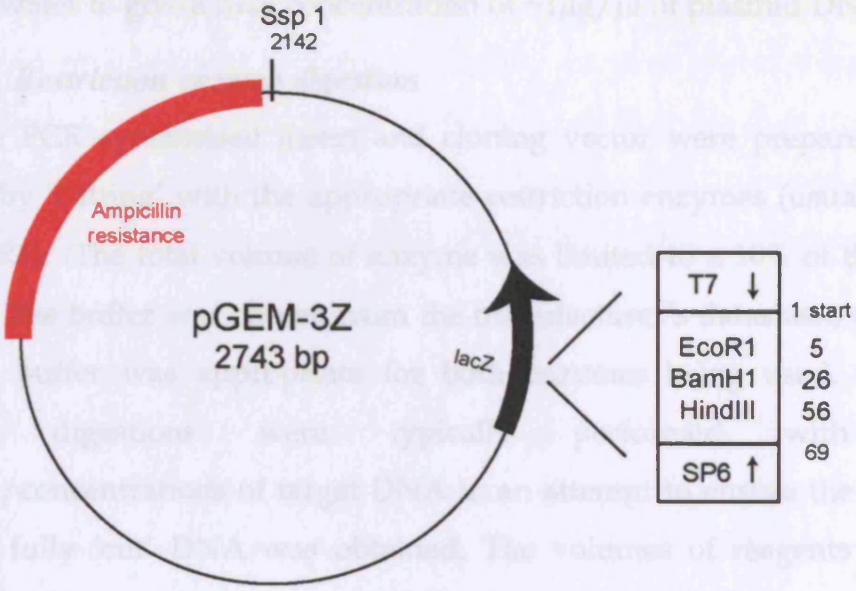


Figure 2-2. pGEM-3Z cloning vector showing ampicillin resistance, multiple cloning site and Ssp1 restriction enzyme site

2.2.3.1.3 Preparation of plasmid DNA - 'midi prep'

A inoculum of E. coli cells containing the pGEM-3Z plasmid (or plasmid with construct inserted) was picked from a glycerol stock kept at -70°C and placed into conical flask with 25ml of 2TY medium and 1 in 100 dilution of ampicillin (stock concentration 5mg/ml). A flask with at least four times the volume of the culture was used. The culture was incubated at 37°C overnight on a rotary shaker. Plasmid DNA was extracted using the QIAGEN-tip 100 plasmid midi-prep kit according to the manufacturer's protocol. This process involves the pelleting of cultured bacterial cells before subjecting them to alkaline lysis. The genomic DNA, cellular proteins, cell debris and SDS are then precipitated from the mixture. The supernatant containing plasmid DNA is purified by centrifuging at ≥ 20,000 x g on two occasions before being passed through an anion exchange column. The plasmid DNA is preferentially bound under conditions of low-salt and appropriate pH. RNA, proteins, metabolites, and other low-molecular-weight impurities are removed by a medium-salt wash, and purified plasmid DNA is eluted in high-salt buffer. Finally, the DNA is

concentrated and desalted by isopropanol precipitation and collected by centrifugation. The yield from a single column was typically resuspended in 200µl of water to give a final concentration of ~1µg/µl of plasmid DNA.

2.2.3.1.4 Restriction enzyme digestion

Both the PCR synthesised insert and cloning vector were prepared prior to ligation by 'cutting' with the appropriate restriction enzymes (usually BamH1 and EcoR1). The total volume of enzyme was limited to ≤ 10% of the reaction volume. The buffer was chosen from the manufacturer's datasheet, taking care that the buffer was appropriate for both enzymes being used. Restriction enzyme digestions were typically performed with several volumes/concentrations of target DNA in an attempt to ensure the maximum yield of fully 'cut' DNA was obtained. The volumes of reagents used in a typical reaction are illustrated in the following table.

Reagent	*Volume /µl*
DNA	Variable (2/5/10)
Enzyme 1	1
Enzyme 2	1
Buffer	2
Water	Variable (14/11/6)
Total	**20µl**

Table 2-12. Typical reaction mixture for Restriction enzyme digestion

The digested inserts were gel purified using the Qiagen gel extraction kit according to the manufacturer's protocol. The principle of this spin column based kit is identical to that of the PCR purification kit described previously (section 2.2.2.6). The products of the vector digestion reaction were assessed by electrophoresis on a 1% agarose gel run out alongside uncut vector to confirm that all vector had been linearised. The cut vector was then subjected to treatment with calf intestinal alkaline phosphatase (CIP) in an attempt to prevent the circular vector re-ligating. De-phosphorylation prevents such self-

ligation whilst permitting the vector to ligate to the phosphorylated termini of another DNA fragment. Briefly, CIP was added to the RE mixture (1unit/pmol vector) and incubated in a water bath at 50°C for one hour. The CIP was then deactivated by adding 1/10 volume 500mM ethylene glycol tetraacetic acid and incubating at 65°C for forty-five minutes. The DNA was then cleaned using phenol:chloroform before ethanol precipitation and resuspension in an appropriate volume of water (for details of this step please see section 2.2.1)

2.2.3.1.5 Ligation reaction

Ligation of the insert and linearised vector was performed using DNA T4 Ligase (1/10 by reaction volume) and 1x Ligase buffer (1/10 volume of 10x stock) in the reaction mixture. The reaction was performed for two hours at room temperature or overnight at 15°C. Approximately 100ng of vector was used with a 10:1 vector:insert ratio by weight. The inserts were between seven to ten times smaller than the pGEM-3Z vector, and this ratio was chosen to provide an excess of linearised vector in the reaction to increase the chance of ligation. The reaction volume was typically 20µl, made up with water.

2.2.3.1.6 Preparation of competent bacteria

Plasmid constructs were initially transformed into E.coli TG-1 cells. Before transformation was performed, a stock of competent cells had to be made. The stock was obtained by inoculating 5mls of 2TY media with a tip culture from glycerol stocks of TG-1 cells and incubating at 37°C overnight on a rotary shaker. The next morning 200µl of this culture was transferred into 20ml of fresh 2TY medium and cultured until the culture medium reached an optical density of 0.3 at 600nm. The culture medium was then chilled on ice for five minutes to arrest growth, before harvesting the cells by centrifuging at 3000rpm for five minutes. The cell pellet was resuspended in 2.5ml of TFB before adding 100µl of DMSO and incubating on ice for five minutes. Dithiothreitol (DTT; 100µl) was then added and the mixture incubated on ice for a further ten minutes. Finally a further 100µl of DMSO was added and the mixture incubated

on ice again for five minutes. The now competent cells were used for transformations in 200µl aliquots.

2.2.3.1.7 Transformation of bacteria with plasmid DNA

Ligated vector and insert were added to 200µl aliquots of freshly prepared competent cells. The volume of ligation reaction added was kept to ≤ 10µl to avoid decreased transformation frequency due to an inhibitory characteristic of the reaction mixture. The mixture was kept on ice for forty minutes before administering a heat-shock by transferring the eppendorf tube to a waterbath at 42°C for two minutes. SOB (1ml) was then added to the heat-shocked cells and the culture incubated at 37°C for thirty minutes on a rotary shaker. Control transformations were also performed with vector alone (positive control), phosphatased-vector and water (negative controls).

2.2.3.1.8 Colony screening

The transformation reaction was assessed by plating 100µl of the transformed cells out onto an LB agar plate with ampicillin. The residual cell culture was then centrifuged for five minutes at 13,000rpm to pellet the remaining cells, part of the supernatant poured away and the remainder used to resuspend the cell pellet. This concentrated cell suspension was then used to make a second back-up plate. The plates were marked and incubated overnight at 37°C.

Following overnight culture the plates were retrieved and the colonies counted. Only bacteria that have acquired antibiotic resistance as a result of transformation with the construct plasmid should grow on the selective plates. Control cultures comprising water or linearised, phosphatased vector and uncut vector provided negative and positive controls respectively. Colonies were circled and numbered, avoiding those smaller colonies on the peripheries where antibiotic concentration may be suboptimal. A selection of colonies was then used to set up cultures for mini-prep analysis. Selective LB agar plates (with ampicillin) were marked into zones using a permanent marker on the underside of the plate. The colony of interest was then 'picked' by touching it with a cooled, flame-sterilised pipette tip. The tip was touched onto the

appropriately marked area of the plate, then back onto the colony again before dropping the tip into 1.5-2ml of 2TY medium. The culture was incubated and agitated at 37°C for a further four to five hours before the plasmid/construct DNA was extracted using the mini-prep method detailed below. The backup plate was cultured overnight at 37°C, before sealing the plate with nescofilm and storing at 4°C.

2.2.3.1.8.1 Mini-prep method

The Wizard SV miniprep kit (Promega, UK) was used according to the manufacturers instructions. This is a spin column system that uses the same principles of alkaline lysis and salt/pH dependent binding of DNA to a column based membrane to the larger scale Qiagen midi-prep kit described earlier. The plasmid DNA was eluted with 100µl of water and stored at -20°C.

2.2.3.1.8.2 Confirming presence of successful construct

The presence of plasmid vector containing the required insert was initially confirmed by double digestion with the two REs used during the cloning procedure. Ten microlitres of the RE digest was then run out on a 1% agar gel stained with ethidium bromide and visualised using UV transillumination. The presence of two bands corresponding to expected size of the vector (2743bp) and the insert (278-497bp) was strongly suggestive a successful cloning procedure. All constructs were then sequenced using primers directed at either the T7 or SP6 promotors adjacent to the cloning sites.

2.2.3.1.9 Long term storage of constructs

Following the successful cloning of the plasmid constructs into readily grown TG-1 cells, residual miniprep DNA was used to transform JM109 cells. These cells are both recombination (recA) and endonuclease (endA) deficient and were chosen to provide a stable host environment for the plasmid constructs. Glycerol stocks were prepared by first preparing a fresh subculture from an overnight tip culture (see above) in selective 2-TY medium. A 500µl aliquot of this second culture was added to 2ml of glycerol in a cryotube and frozen in an insulated box at -70°C before long-term storage in liquid nitrogen.

2.2.3.2 RNA in situ hybridisation

2.2.3.2.1 In vitro synthesis of riboprobes

Digoxigenin-UTP labelled riboprobes were synthesised from the plasmid constructs using a proprietary *in vitro* transcription kit (Roche Diagnostics Ltd.) according to the manufacturer's instructions. Linearised transcription vector was prepared by RE digestion at either BamH1 or EcoR1 sites. The product was gel purified using the Qiagen gel extraction kit according to the manufacturers protocol. The T7 or SP6 promoters were then used to initiate transcription with RNA polymerase leading to the production of anti-sense or sense probes (with reference to mRNA target) respectively. Digoxigenin labelled UTP was incorporated into the RNA transcript approximately every 20-25 nucleotides. Labelled probes were ethanol precipitated, the RNA pelleted by centrifugation and the pellet resuspended in dimethlyldicarbonate-treated water. RNase inhibitor was added to inhibit possible contaminating RNases.

2.2.3.2.2 Dot-blot hybridisation of digoxigenin-labelled probes

The concentration of probe was assessed by dot-blot hybridisation of serial dilutions of the RNA probe. Briefly, a nitrocellulose membrane was marked with a grid. Each row of the grid was used to test a separate RNA probe. One microlitre of the neat probe was placed in the first column and a ten-fold serial dilution (with water) in each of the subsequent columns down to a dilution of 1 in 100,000. A UV-crosslinker was used to bind the nucleic acid to the membrane. After washing to remove non-bound probe (1 minute in Buffer 1) and blocking non-specific signal (30 minutes in Buffer 2) the membrane was incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (30 minutes; 1 in 500 dilution in Buffer 2). The membrane was then subjected to stringency washing (Buffer1, 2x 5 minutes and Buffer 3, 1x 5 minutes). Detection of bound antibody was performed by incubating the membrane in NBT/BCIP colour change solution (1 in 50 dilution in Buffer 3) in a dark container for up to 4 hours. The colour change reaction was ended by washing the filter in distilled water and the filter was then inspected. After drying, filters were stored in a sealed polythene bag.

2.2.3.2.3 In situ hybridisation protocol

The RNA ISH protocol requires steps to be performed on three consecutive days. All steps were performed at room temperature in Coplin jars unless otherwise stated. Technical assistance for RNA ISH was provided by Ms. A. Nicholson, Division of Virology, University of Cambridge.

2.2.3.2.3.1 Prehybridisation

Paraffin-embedded tissue sections (7μm) were cut and mounted on APES coated slides (Mrs. B. Haynes, Department of Histopathology, University of Cambridge). Slides for RNA ISH were first dewaxed by soaking in Xylene for two washes of two minutes each before rehydrating using a graded series of alcohols (100%, 100%, 70%, 50% and 30%; 5 mins x2 for the 100% alcohol, then two minutes each subsequent step). Following gradual rehydration slides were washed in distilled water for two minutes before a final five minute wash in phosphate-buffered saline (PBS). The sections were then fixed, to preserve tissue morphology and mRNA, using 4% paraformaldehyde in PBS for five minutes before washing in PBS (4 x 2 minutes). The slides were then incubated in pre-warmed proteinase K buffer for three minutes at 37°C before treating with proteinase K solution (proteinase K buffer plus proteinase K (20μg/ml)) for exactly ten minutes at 37°C. The sections were then washed in PBS (4 x 2 minutes) and re-fixed in 4% paraformaldehyde in PBS for five minutes. After paraformaldehyde fixation, the slides were washed in PBS again (4 x 2 minutes) and acetylated to reduce non-specific background signal by treatment with acetic anhydride/triethanolamine solution for ten minutes. The sections were then washed in PBS(4 x 2 minutes) and dehydrated using a graded alcohol series (30%,50%,70%,100% and 100%; 1 minute each and 5 minutes for each of the 100% alcohol steps).

2.2.3.2.3.2 Hybridisation

The slides were then allowed to air dry prior to application of the probe to be tested. Ten micolitres of probe (1-1.5 in 10 dilution of probe in hybridisation buffer (Sigma)) was applied to each site before covering with a coverslip. The

slides were placed into a prewarmed, humidified box and incubated overnight at 37°C.

2.2.3.2.3.3 Stringency washes and detection

The coverslips were removed by dipping slides into 2x SCC / 0.1% SDS at 55°C and the slides were washed in 2x SCC / 0.1% SDS at 55°C for ten minutes, ensuring that the temperature in the coplin remained stable by insulating it in a polystyrene box and placing this on a magnetic stirrer. Unhybridised RNA was then removed by direct application of 2mg/ml RNases in 5x SSC and incubation at 37° C for fifteen minutes. The slides then underwent further stringency washing in 1x SSC / 0.045% SDS at 65°C (2x 20 minutes). The slides were subsequently soaked in Buffer 2 for thirty minutes before incubation for thirty minutes with anti-digoxigenin antibody conjugated to alkaline phosphatase (1 in 500 dilution in Buffer 2). Following antibody detection, the slides were washed twice in Buffer 1 for fifteen minutes each time and soaked in Buffer 3 for thirty minutes. Antibody-probe binding was detected by application of NBT/BCIP colour change solution (125µl in 5ml Buffer 3) and incubation overnight in a dark box. The following day, the colour change reaction was stopped by immersing the slides in 1x TE at pH8.0. The slides were then rinsed twice in PBS and water respectively before counterstaining with Eosin and mounting with an aqueous mountant (Faramount, Dako Ltd.).

2.2.3.2.3.4 Controls

Probing with the sense RNA probe provided an internal negative control for each hybridisation, whilst the use of a known keratin mRNA probe (keratin 10 or 14, supplied by Dr J.C. Sterling, University of Cambridge) and sections from a proliferative wart served as a positive control for the technique.

2.2.4 Comparative genomic hybridisation (CGH)

CGH was performed on DNA extracted from cells obtained by microdissection of appropriately prepared frozen sections (section 2.2.1).

2.2.4.1 Degenerate oligonucleotide-primed PCR (DOP-PCR)

Genomic 'test' and normal 'control' DNA was amplified and differentially labelled using DOP-PCR (for a detailed discussion of this technique, please see section 1.2.3.2.2.3.1)

2.2.4.1.1 Primary DOP-PCR

The primary amplification of genomic DNA was performed in a 50µl reaction using a PCR buffer kit comprising water, Buffer D and dNTP (Invitrogen), together with the 6MW primer (5' CCG ACT CGA GNN NNN NAT GTG G 3'), high-concentration Taq polymerase (HT-Biotechnologies) and W1-detergent (Sigma-Aldrich Ltd.). The reaction mixture and cycling conditions are described in the following tables:

Reagent	*Stock concentration*	*Reaction concentration*	*Volume (µl)*
Microdisected lysate	n/a	n/a	5
Buffer D	X5	X1	10
dNTP	2mM	0.2 mM	5
W1 Detergent	X20	X1	2.5
6MW primer	20 µM	2 µM	5
Taq polymerase	15 U/µl	0.15 U/µl	0.5
Water	22	n/a	22
		TOTAL	**50µl**

Table 2-13. Primary DOP-PCR reagents

Step		*Temparature / ℃*	*Time / min*	*Ramp rate*	*cycles*
	Denaturing	95	9		
Low specificity cycles	Denaturing Annealing Extension	94	1.5 1 sec 3	0.23°C/s	X5
High specificity cycles	Denaturing	94	0.5		
	Annealing Extension	62 72	1 1		X30
	Extension	72	7		
	Hold	4	indefinite		

Table 2-14. Primary DOP-PCR cycling conditions

The suitability of amplified DNA was checked by gel electrophoresis using 5µl of reaction mixture and a 1% agarose gel.

2.2.4.1.2 Secondary DOP-PCR

The amplified test and control DNA was then labelled by the incorporation of hapten conjugated dUTP using a second round of PCR with the 6MW primers. The second round of PCR was carried out under high specificity conditions (Table 2-16). The reaction mixture differs from the primary DOP-PCR in that the concentration of dTTP in the dNTP mixture was reduced to 1.6mM and replaced with labelled dUTP. Digoxigenin-11-dUTP was used to label the test DNA and biotin-16-dUTP to label the control DNA (Boehringer Mannheim). The higher annealing temperature of 62°C was used for all amplification cycles in the secondary DOP-PCR.

Reagent	*Stock concentration*	*Reaction concentration*	*Volume /µl*
Primary DOP product	n/a	n/a	2.5
TAPs buffer	X10	X1	5
dNTP	2mM A,G and C 1.6mM T	0.2 mM 0.16mM	5
dUTP-labelled	1mM	0.02mM	2
W1 Detergent	X20	X1	2.5
6MW primer	20 µM	2 µM	5
Taq polymerase	15 U/µl	0.15 U/µl	0.5
Water	27.5	n/a	27.5
		TOTAL	**50µl**

Table 2-15. Reagents for secondary DOP-PCR (labelling)

Step	*Temparature / ℃*	*Time / min*	*cycles*
Denaturing	95	5	
Denaturing	94	0.5	
Annealing	62	1	29
Extension	72	1	
Extension	72	7	
Hold	4	Indefinite	

Table 2-16. Secondary DOP-PCR cycling conditions

2.2.4.2 Preparation of probe

Approximately 150ng of each labelled DNA was added to 15µg of Cot-1 placental DNA (GibcoBRL) and the resulting DNA mixture precipitated with 0.1 volumes 3M sodium acetate (pH5.2) and 2.5 volumes of absolute ethanol. The precipitate was resuspended in 16µl CGH hybridisation buffer (50% formamide, 10% dextran sulphate, 2xSSC, 40 mM sodium phosphate buffer (pH7) and 1x Denhardts solution (Sigma)). The probe was then denatured by

incubation at 65°C for 10 minutes before undertaking a pre-annealing step of 37°C for 30 minutes to facilitate competitive suppression of the highly repetitive sequences by the placental Cot-1 DNA.

2.2.4.3 Hybridisation

Normal male metaphase slides (Abbott Diagnostic Division) were denatured at 73 +/- 1 °C in prewarmed 70% formamide / 2xSSC for 4 minutes. Denatured slides were immediately cooled by placing into ice-cold 70% ethanol, before dehydrating using a graduated series of ethanols (70%, 90% and 100% ethanol for 5 minutes each). The dehydrated slides were then air-dried and 15µl of the prepared probe applied to the relevant metaphase site. The site was covered with a glass coverslip and sealed using a suitable rubber cement. The slide was then placed in a pre-warmed incubation box at 37°C for at least 48 hours.

2.2.4.4 Stringency washing and Detection

The slides were first incubated in 2xSSC at 37°C to soften the adhesive and allow careful removal of the glass coverslip. Non-specific probe hybridisation was removed by washing in 50% formamide / 1xSSC at 43°C for 5 minutes twice, followed by two further washes in 0.1x SSC at 43°C for 5 minutes each. Slides were then incubated in 4xSST for 3 minutes. To reduce non-specific binding of detection reagents, 100µl of 3% bovine serum albumin (BSA) / 4xSST was added to each site and the slides incubated for ~30 minutes. Excess blocking agent was then removed by three washes (3 minutes each) in 4xSST at 37°C.

Digoxigenin-labelled test probe was detected using a 1 in 200 dilution of monoclonal mouse anti-digoxigenin Fab fragments, conjugated to FITC (Roche Diagnostics ltd.). Biotin-labelled control probe was detected using a 1 in 400 dilution of streptavidin conjugated to Cy3 (Amersham Biosciences ltd). Dilution was with 1% BSA in 4xSST and performed in a light-tight pre-warmed incubation box at 37°C for 90 minutes.

Unbound streptavidin or antibody was removed by three washes of 3 minutes each at 37°C. Excess liquid was removed from the slide and the slides mounted

using an anti-fade solution containing 0.4μM DAPI (Vectashield with DAPI, Vector laboratories).

2.2.4.5 CGH Imaging

Imaging and capture of CGH data was performed on an Axioplan II fluorescence microscope (Zeiss) equipped with the appropriate excitation and emission filters (Chroma Technology, Ltd) and a Sensys CCD camera (Roper Scientific) controlled by a Power Macintosh 7600 computer (Apple Computers Int.) running Smartcapture VP software (Digital Scientific).

2.2.4.6 CGH Analysis

At least ten representative metaphases were captured from each case and the results combined to produce and average fluorescence ratio along the length of each chromosome using QUIPS karyotyping and CGH interpretation software (Abbott Diagnostic Division - Vysis). Increases and decreases in DNA sequence copy number were defined to test by reference ratios of > 1.15 and < 0.85 respectively. Control experiments included template negative DOP-PCR reactions, hybridisation of DNA from normal vulval epithelium to normal control DNA, hybridisation from donor peripheral lymphocytes to normal control DNA and control DNA versus control DNA hybridisation. The reference ranges were constructed after a number of these normal to normal hybridisations showed that the mean fluorescence ratio stayed between 0.85 and 1.15 along all chromosomes.

2.2.5 Vaccination and the measurement of immune response to HPV

Patients were recruited, vaccinated and histological, serological and haematological samples collected by the author at Addenbrooke's NHS trust. Cambridge. Vaccine and HPV-specific immune responses were measured in collaboration with two groups:

- Mr. C. Boswell, Xenova Group plc, Cambridge, UK
- Dr. S. van der Burg, Tumour immunology group, University of Leiden, the Netherlands

Details of the methods used to assess immune responses to vaccination are included in the following section.

2.2.5.1 Vaccination by dermal scarification

Live recombinant vaccinia virus was introduced into the patient by scarification of the skin of the upper arm. 50 µl of virus (~2.5 x 10^6 pfu) was applied in a fluid droplet over intact skin and the skin scratched superficially with a bevelled needle through the fluid which was then allowed to dry before covering with a waterproof dressing (Opsite, Smith & Nephew). The dressing was changed twice a week for approximately 4 weeks until the scab that formed as a result of the virus-induced inflammation separated and the vaccination site was healed. Samples for virological detection were taken from the surface of the dressing every week before its removal to test for the risk of possible contamination of the environment.

2.2.5.2 Anti-vaccinia ELISA

The anti-vaccinia ELISA used in this work was performed by Mr C. Boswell, Xenova group plc, Cambridge. Whole blood was collected in one 7.5ml S-Monovette tube (Sarstedt) at days 0, 28, 56 and 84. Serum was separated and stored at -20°C until required for analysis. Vaccinia-specific IgG was measured in patient sera by ELISA using Wyeth strain vaccinia-infected Vero cell lysates as antigen and mock-infected Vero cell lysates as the control (Borysiewicz *et al.*,

1996). The titre at OD 0.5 was determined using the line of best fit from the graph of the log sample dilution vs. sample Wyeth OD minus sample Vero OD.

2.2.5.3 ELISPOT Technique

Whole blood was collected in S-Monovette 8.5ml CPDA tubes (Starstedt) and PBMC were isolated by density centrifugation on the same day. Cells were cryopreserved in vapour phase nitrogen until required.

2.2.5.3.1 Analysis of HPV16-specific T-cell responses with overlapping E6- and E7-peptides.

IFNγ ELISPOT assays were performed by Dr. S.V. van der Burg of the Tumour Immunology Group, Unversity of Leiden, the Netherlands as has been described elsewhere (van der Burg *et al.*, 2001b). Briefly, PBMC were thawed and seeded at a density of $2x10^6$ cells/well of a 24-well plate (Costar, Cambridge, MA) in 1ml of ISCOVE's medium (Gibco) enriched with 10% human AB serum, in the presence or absence of indicated E6 and E7 peptide pools. As a positive control, PBMC were cultured in the presence of a memory recall mix (MRM), consisting of a mixture of tetanus toxoid (0.75LF/ml final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5g/ml; generously donated by Dr P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and *Candida albicans* (0.005%, HAL Allergenen Lab. Haarlem, The Netherlands). The peptides used spanned the HPV 16 E6 and E7 protein and consisted of fifteen E6 and nine E7 overlapping 22-mer peptides. Peptides were used in pools of 4-5 peptides at a concentration of 5g/ml/peptide. The peptides, as indicated by their first and last amino acid, were used in the following pools: E6-I: 1-22, 11-32, 21-42, 31-52; E6-II: 41-62, 51-72, 61-82, 71-92; E6-III: 81-102, 91-112, 101-122, 111-132; E6-IV: 111-132, 121-142, 131-152, 137-158; E7-I: 1-22, 11-32, 21-42, 31-52; E7-II: 41-62, 51-72, 61-82, 71-92, 77-98 (van der Burg *et al.*, 2001b). Following four days of incubation at 37°C, PBMC were harvested, washed and seeded in four replicate wells at a density of 10^5 cells/well in 100µl of ISCOVE's medium (Gibco) enriched with 10% FCS in a multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with

an INFγ catching antibody (Mabtech AB, Nacha, Sweden). The ELISPOT was otherwise performed according to the instructions of the manufacturer (Mabtech). The number of spots were analysed with a fully automated computer-assisted video imaging analysis system (Carl Zeiss Vision). The background number of spots in the medium controls was 2.3+/-2 spots per 50,000 PBMC. Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium-only control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be increased compared to non-responders when specific T-cell frequencies were >1/10,000 (van der Burg *et al.*, 2001b). T-cell frequencies were considered to be boosted by the vaccine when they were at least 3 fold higher than before vaccination.

2.2.5.3.2 Analysis of CD8+ T-cell responses with HLA class I restricted HPV epitopes.

CD8+ responses were investigated by Dr. S.V. van der Burg of the Tumour Immunology Group, Unversity of Leiden, the Netherland. ELISPOT plates (MAIPS410, Millipore) were prepared with the following antigens in triplicate wells: PHA 1μg/ml; synthetic peptides (ISL) of HPV 16 $E6_{29\text{-}38,\ 59\text{-}67,\ 80\text{-}88}$ and $E7_{11\text{-}20,\ 82\text{-}90,\ 86\text{-}93}$ (Kast *et al.*, 1994; Ressing *et al.*, 1995) influenza virus $M1_{58\text{-}66}$, and EBV $BMLF1_{280\text{-}288}$ all at 25μg/ml or inactivated vaccinia virus and control uninfected Vero cell lysate 1:2500. Patient PBMC from each time point were thawed from cryopreserved stocks and resuspended in medium (RPMI (GibcoBRL), 10% AB serum (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine (all GibcoBRL)) at $5x10^6$/ml. PBMC were then added to wells in triplicate, $2.5x10^5$ to the PHA wells and $5x10^5$ to each of the remaining wells containing antigen or medium alone. Following overnight incubation and washing, biotinylated monoclonal antibody to IFNγ (7-B6-1-biotin, Mabtech) was added, followed by streptavidin-conjugated horseradish peroxidase (Mabtech). The assay was developed according to the manufacturer's instructions (Mabtech). A positive response to antigen was defined as greater than 20 spots (IFNγ producing cells) per 10^6 PBMC in response to antigen

(following subtraction of background). A positive response following vaccination was recorded if the frequency of IFNγ producing cells post-vaccination was greater than 2 times the pre-vaccination response to an antigen.

2.2.6 Suppliers

The suppliers used for the reagents detailed above are listed in the following table.

Supplier	*Contact details*	*Web address*
Abbott Diagnostic division (includes Abbott –Vysis)	Abbott House Norden Road Maidenhead Berks SL6 4XF UK	www.abbotuk.com www.vysis.com
Agar Scientific Ltd.	66a Cambridge Road Stansted Essex CM24 8DA UK	www.agarscientific.com
Ambion (Europe) Ltd.	Spitfire Close Ermine Business Park Huntingdon Cambridgeshire PE29 6XY UK	www.ambion.com
Amersham Biosciences UK	Amersham Place Little Chalfont Buckinghamshire HP7 9NA UK	www.apbiotech.com
Apple Computer Int.	Hollyhill Industrial Estate Cork Republic of Ireland	www.apple.com/uk/
BDH Laboratory Supplies	Poole Dorset DH15 ITD UK	www.bdh.com
Bioquote Ltd.	The Raylor Centre James Street York YO10 3DW UK	www.bioquote.com
Chroma Technology Corp.	10 Imtech Lane PO box 489	www.chroma.com

Supplier	*Contact details*	*Web address*
	Rockingham VT05101 USA	
Dako Ltd.	Denmark House Angel Drive Ely Cambs CB7 4ET UK	www.dako.co.uk
Digital Scientific Ltd.	Sheraton House Castle Park Cambridge CB3 0AX UK	www.digitalscientific.co.uk
Fisher Scientific	Bishop Meadow Road Loughborough Leicestershire LE11 5RG UK	www.fisher.co.uk
HT Biotechnology Ltd.	Ditton Walk Cambridge CB5 8QD UK	www.stratech.co.uk
Invitrogen life technologies	3 Fountain Drive Inchinnan Business Park Paisley UK	www.invitrogen.com
Merck Ltd.	Merck House Poole Dorset BH15 1TD UK	www.merck-ltd.co.uk
Nalge (Europe) Ltd.	Unit 1a, Thorne Business Park Hereford HR2 6JS UK	www.nalgenelabware.com
PAA Laboratories ltd.	1 Guard Avenue Houndstone Business Park Yeovil Somerset BA22 8YE UK	www.paa.at
Promega UK	Delta House Chilworth Science Park Southampton SO16 7NS UK	www.promega.com/uk/

Supplier	*Contact details*	*Web address*
Qiagen Ltd.	Boundary Court Gatwick Road Crawley West Sussex RH10 2AX UK	www.qiagen.com
Roche Diagnostics Ltd.	Bell Lane Lewes East Sussex BN71LG UK	www.biochem.roche.com
Roper Scientific	c/o Universal Imaging Corporation Ltd. PO Box 1192 43 High Street Marlow Buckinghamshire SL7 1GB UK	www.roperscientific.com
Sarsted Ltd.	68 Boston Road Beaumont Leys Leicester LE4 1AW UK	www.sarstedt.com
Sigma-Aldrich	Fancy Road Poole Dorset BH12 4QH UK	www.sigma-aldrich.com
STARLAB (UK) Ltd.	Unit 4, Tanners Drive Blakelands Milton Keynes MK14 5NA UK	http://www.starlab.de
Vector laboratories Ltd.	3 Accent Park Bakewell Road Orton Southgate Peterborough PE2 6XS UK	www.vectorlabs.com

Table 2-17. List of suppliers

Chapter 3 Human papillomavirus infection and vulval intraepithelial neoplasia

3.1 Introduction

The association of oncogenic HPVs and their role in the development of neoplasia of the lower genital tract has been discussed in detail (section 1.2.1). The action of high-risk E6 and E7 proteins appears central to this process. Expression of these oncoproteins may become increasingly deregulated through the loss of transcriptional control that occurs during integration of viral DNA into the host genome (section 1.2.1.5). Viral integration has been extensively studied in cervical neoplasia, but data regarding the frequency and importance of such integration in both VIN and SCC of the vulva are lacking.

VIN remains a frustrating therapeutic challenge to the clinician. Current therapies are poorly tolerated and recurrence rates are high. In this respect, the association between HPV and VIN may provide a therapeutic opportunity. Viral gene products are attractive as targets for novel immunotherapies in HPV-associated VIN. To ensure that such therapies are directed at appropriate gene products, it is necessary to better understand the pattern of HPV gene expression within target lesions.

In this chapter, the use of sensitive PCR techniques to investigate the incidence of HPV infection and the physical state of the virus in women with vulval neoplasia will be described. In the methods section, the optimisation of the PCR systems employed to assess both the presence and physical state of HPVs will be detailed. The results section describes the use of these PCR systems to investigate a cohort of patients with vulval neoplasia, as well as the further investigation of gene expression in HPV 16-positive individuals using the technique of RNA *in situ* hybridisation.

3.2 Methods

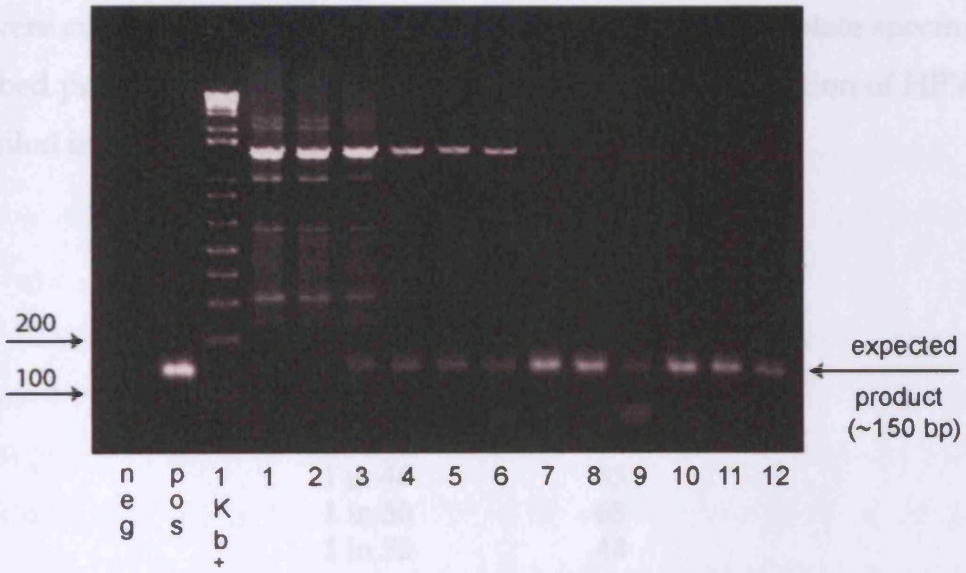
3.2.1 Optimising and validating PCRs for assessing the presence and physical state of human papillomaviruses

The HPV detection strategy used throughout this project is detailed in the methods chapter (section 2.2.2.6). Briefly both GP5+/6+ (de Roda Husman *et al.*, 1995) and MY 09/11 (Manos *et al.*, 1989) primer systems were used independently and in a nested fashion. The E2 gene is commonly disrupted during integration (Schwarz *et al.*, 1985; Baker *et al.*, 1987; Dürst *et al.*, 1987). Viral integration was therefore assessed using PCR to demonstrate the presence or absence of an intact E2 gene as a surrogate marker for integration (Das *et al.*, 1992). All PCRs were optimised for the specific reagents and apparatus used in this thesis. Modifications of the standard PCR protocols and their validation will be detailed in the following sections.

3.2.1.1 An annealing temperature of 50°C permits reduction in non-specific target DNA amplification with the GP5+/6+ primer system

The GP5+/6+ PCR system relies on the use of a low annealing temperature of 40°C to allow sufficient annealing between consensus primers and template (de Roda Husman *et al.*, 1995). Such low-stringency annealing conditions promote high-sensitivity detection of multiple HPV types, allowing for a degree of mismatch base pairing between the consensus primer and a variety of templates. However, the use of low annealing temperatures and high numbers of amplification cycles (40 cycles) tends to reduce PCR specificity and may result in the amplification of non-target DNA. As has been detailed elsewhere (section 2.2.2.6) HPV typing in this thesis was performed by the purification and sequencing of the PCR products obtained from the GP5+/6+ (and MY09/11) systems. It was therefore essential to reduce the levels of non-specific amplification to produce a 'pure' PCR product that was suited to direct sequencing. To investigate the effect of varying annealing temperatures on non-specific amplification by the GP5+/6+ primers a series of PCR reactions were performed using a known amount of HPV DNA from CaSki cells (equivalent to

~200 copies of HPV16) mixed with HPV-negative DNA from human placental tissue (~300ng). The PCR was run on a block-thermocycler with a gradient-block that allowed the reaction to be performed using multiple different annealing temperatures within a single PCR experiment. The PCR mix was prepared as detailed in the methods section using a 'master-mix' approach to minimise variation between reaction tubes. Ten microlitres of reaction product were run out on a 1.5 % agarose gel stained with ethidium bromide and visualised using UV transillumination (Figure 3-1. Optimising annealing temperature for GP5+/6+ PCR with gradient PCR). A control reaction in which water replaced the target DNA was used as a negative control, whilst a reaction with the CaSki-extracted DNA alone was used a positive control.



Lane	1	2	3	4	5	6	7	8	9	10	11	12
Temp / °C	40.1	40.5	41.3	42.7	44.4	46.4	48.3	50.3	52.6	54.0	54.7	55.2

Figure 3-1. Optimising annealing temperature for GP5+/6+ PCR with gradient PCR

The negative and positive controls were appropriate. A product of ~150bp was visible in each of the lanes representing the range of annealing temperatures tested (40.1°C to 55.2°C). The product corresponds to the amplicon expected from the GP5+/6+ primer system. At lower annealing temperatures multiple bands representing non-specific target DNA amplification were visualised. As

expected, the amount of non-specific amplification reduced with increasing annealing temperature. When the annealing temperature approached 45°C, five degrees below the highest Tm for the primers this non-specific amplification had reduced considerably and by 48-50°C a solitary product was evident (lanes 7 and 8). This result was reproducible.

3.2.1.2 The sensitivity of HPV detection (type 16) using GP5+/6+ remains acceptable under these new conditions

Although the increase in PCR specificity was desirable, such a significant increase in annealing temperature might be expected to have a deleterious effect on PCR sensitivity. The sensitivity of the GP5+/6+ PCR performed at the new annealing temperature of 50°C was therefore assessed. CaSki and SiHa cells were cultured and used to set up a series of control template specimens as described previously (section 2.2.2.2). The assumed concentration of HPV DNA is detailed in the table below.

SiHa cells	
Dilution	***Copies / 2μl***
Neat	3,400
1 in 10	340
1in 20	170
1 in 40	85
1 in 50	68
1 in 70	48

Table 3-1. Serial dilutions of DNA extracted from HPV-positive SiHa cell line. Estimated number of copies per 2μl load specified.

Ten microlitres of PCR product was visualised by UV transillumination on a 1.5% agarose gel stained with ethidium bromide. Product of the expected amplicon size (~150bp) was visualised to a dilution of 1 in 70 suggesting that the sensitivity threshold for the modified PCR reaction was at worst 48 copies of HPV 16 DNA per 50μl reaction mixture (Figure 3-2).

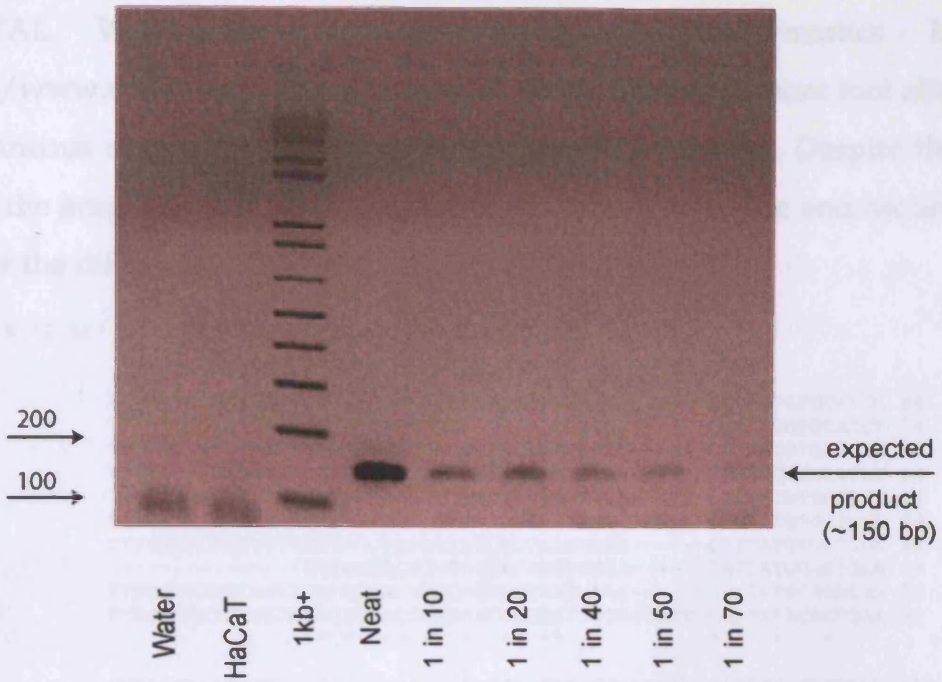


Figure 3-2. Sensitivity of GP5+/6+ primer system established using serial dilutions of SiHa cell DNA. Gel image has been inverted to facilitate visualisation of weaker bands. Product just visible at 1 in 70 dilution suggesting a detection threshold ≥ 48copies of HPV 16 per 50µl PCR reaction.

Control SiHa DNA was prepared from a known number of cells prepared in tissue culture. The estimation of the number of copies of HPV 16 was based on the assumption that SiHa cells contain one copy of HPV 16 DNA and that DNA extraction from the cells lines was 100% efficient. This is unlikely to be the case. The control DNA would therefore be expected to contain fewer copies of HPV 16 than was estimated and the PCR is probably more sensitive than indicated by this experiment. It would appear that increasing the annealing temperature still permits the sensitive detection of HPV 16 DNA.

3.2.1.3 Sequence variation in target amplicon should permit the distinction of genital HPV types by genotyping of the GP5+/6+ PCR product

The purification, sequencing and virus typing of the GP5+/6+ PCR products is detailed in the method sections (section 2.2.2). Although this method of HPV typing has been used by others (Rosenthal *et al.*, 2001) a multiple alignment of the GP5+/6+ products from a variety of HPV types was performed using the

CLUSTAL W service at the European Bioinformatics Institute (http://www.ebi.ac.uk/, Thompson *et al.*, 1994). This alignment tool allows the simultaneous comparison of multiple nucleotide sequences. Despite the small size of the amplicon, significant variations between both size and sequence are seen for the differing HPV types.

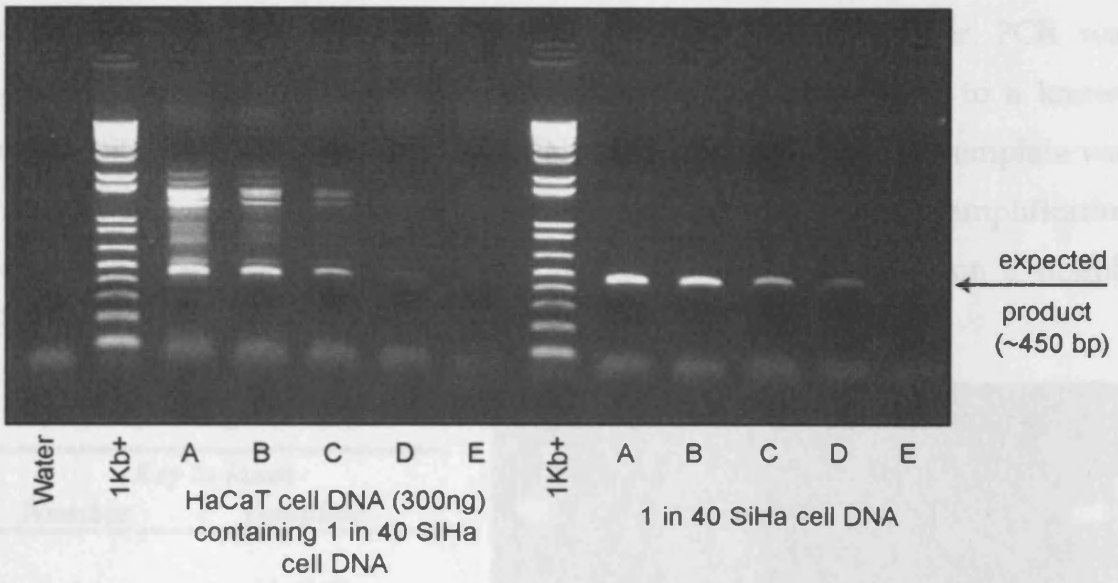
```
CLUSTAL W (1.82) multiple sequence alignment


gp6b            TTTGTTACTGTGGTAGATACCACACGCAGTACCAACATGA------CATTATGTGCATCC 54
gp11            TTTGTTACTGTGGTAGATACCACACGCAGTACAAATATGA------CACTATGTGCATCT 54
gp16            TTTGTTACTGTTGTTGATACTACACGCAGTACAAATATGT------CATTATGTGCTGCC 54
gp18            TTTGTTACTGTGGTAGATACCACTCCCAGTACCAATTTAA------CAATATGTGCTTCT 54
gp45            TTTGTTACTGTAGTGGACACTACCCGCAGTACTAATTTAA------CATTATGTGCCTCT 54
gp31            TTTGTTACTGTGGTAGATACCACACGTAGTACCAATATGT------CTGTTTGTGCTGCA 54
gp52            TTTGTCACAGTTGTGGATACCACTCGTAGCACTAACATGA------CTTTATGTGCTGAG 54
gp58            ---------------GATACCACTCGTAGCACTAATATGA------CATTATGCACTGAA 39
gp33            TTTGTTACTGTGGTAGATACCACTCGCAGTACTAATATGA------CTTTATGCACACAA 54
gp8             TTTGTCACTGTGGTAGACAACACGCGAAACACCAATTTTAGTATTTCAGTTTACACTGAA 60
                                ** *  ** *  *  ** **  *        *  * *   *

gp6b            GTAACTACATCTT---CCA---CA---TACACCAATTCTGATTATAAAGAGTACATGCGT 105
gp11            GTGTCTAAATCTG---CTA---CA---TACACTAATTCAGATTATAAGGAATACATGCGC 105
gp16            ATATCTACTTCAGAAACTA---CA---TATAAAAATACTAACTTTAAGGAGTACCTACGA 108
gp18            ACACAGTCTCCTGTACCTGGGCAA---TATGATGCTACCAAATTTAAGCAGTATAGCAGA 111
gp45            ACACAAAATCCTGTGCCAAGTACA---TATGACCCTACTAAGTTTAAGCAGTATAGTAGA 111
gp31            ATTGCAAACAGTGATACTA---CA---TTTAAAAGTAGTAATTTTAAAGAGTATTTAAGA 108
gp52            GTTAAAAAGGAAA---GCA---CA---TATAAAAATGAAAATTTTAAGGAATACCTTCGT 105
gp58            GTAACTAAGGAAG---GTA---CA---TATAAAAATGATAATTTTAAGGAATATGTACGT 90
gp33            GTAACTAGTGACA---GTA---CA---TATAAAAATGAAAATTTTAAAGAATATATAAGA 105
gp8             AATGGGGAACTTAAGAACATCACAGACTATAAATCAACCCAGTTCAGAGAATATCTGAGA 120
                                  *   *            * *  *   * **     *

gp6b            CATGTGGAAGAGTATGATTTACAATTTATTTTTC 139
gp11            CATGTGGAGGAGTTTGATTTACAGTTTATTTTTC 139
gp16            CATGGGGAGGAATATGATTTACAGTTTATTTTTC 142
gp18            CATGTTGAGGAATATGATTTGCAGTTTATTTTTC 145
gp45            CATGTGGAGGAATATGATTTACAGTTTATTTTTC 145
gp31            CATGGTGAGGAATTTGATTTACAATTTATATTTC 142
gp52            CATGGCGAGGAATTTGATTTACAATTTATTTTTC 139
gp58            CATGTTGAAGAATATGACTTACAGTTTGTTTTTC 124
gp33            CATGTTGAAGAATATGATCTACAGTTTGTTTTTC 139
gp8             CATGTAGAAGAATATGAAATTTCCCTCATATTAC 154
                ****  ** ** * ***  *     *  * ** *
```

Figure 3-3. CLUSTAL W multiple alignment for GP5+/6+ amplicons. Data shown for from HPV types 6b, 11, 16, 18, 31, 22, 45, 52, 58 and the EV type 8.

3.2.1.4 Non-specific amplification at low-target concentration with MY09/11 system is reduced by increasing the annealing temperature

The MY09/11 primer system was used to reassess samples negative by GP5+/6+ PCR and as part of the nested PCR protocol (section 2.2.2.5.1). A thermal cycler with a gradient block was used to assess the optimum annealing temperature in order to minimize non-specific amplification at low target concentration. The method used was similar to that used in optimising the

GP5+/6+ PCR system except that HPV negative DNA from HaCaT cells (~300ng) was used to provide a background signal in the presence of a 1 in 40 dilution of SiHa cells (~85 copies per PCR). The Tm of the primer sets MY09 and My11 are 56°C and 58°C respectively. A range of temperatures from 53°C upwards were assessed. A parallel PCR using HPV positive template DNA from SiHa cells (~85 copies of HPV16 per reaction) was performed to assess sensitivity at the various annealing temperatures tested. Ten microlitres of PCR product were electrophoresed on a 1.5% agarose gel and visualised with UV light (Figure 3-4).



Lane	A	B	C	D	E
Annealing temperature / °C	53.1	54.7	57.0	58.9	61.3

Figure 3-4. Gradient PCR with MY09/11 primer set to establish optimal annealing temperature. HaCaT/SiHa DNA mix used to assess non-specific amplification at low template concentrations. SiHa DNA used to assess sensitivity simultaneously. 1 in 40 dilution of SiHa cells equates to approximately 85 copies of HPV 16 per reaction mixture.

The 'water template' control was appropriately negative. Marked non-specific amplification with a resultant 'ladder' of PCR products was seen at low annealing temperatures. The amount of such amplification decreased with

increasing annealing temperature. The optimum annealing temperature to achieve low non-specific amplification without a marked reduction in sensitivity lies between 54.7 and 57°C. Given the melting temperatures of the primers an annealing temperature of 55°C was used for further experiments.

3.2.1.5 Non-specific amplification produces a band close to the expected amplicon size from HPV-negative HaCaT DNA

A non-specific ladder pattern was seen with initial PCR using the MY09/11 primer system and known HPV-negative DNA from HaCaT cell lines (Figure 3-4). GP5+/6+ PCR produced no such false-positive band with HaCaT DNA suggesting that the DNA was truly HPV-negative. A further PCR was performed to compare such apparent non-specific amplification to a known 'true-positive' signal from SiHa cell DNA. The HaCaT cell DNA template was diluted with water in a serial fashion to establish the effect on this amplification pattern. Ten microlitres of PCR product were electrophoresed on a double length 1.5% agarose gel and visualised with UV light (Figure 3-5).

Key to lanes	
Number	***Template***
1	HaCaT
2	1 in 40 SiHa
3	1in 5 HaCaT
4	1 in 40 SiHa
5	1 in 10 HaCaT
6	1 in 40 SiHa
7	1 in 50 HaCaT
8	1 in 40 SiHa
9	1 in 100 HaCaTs

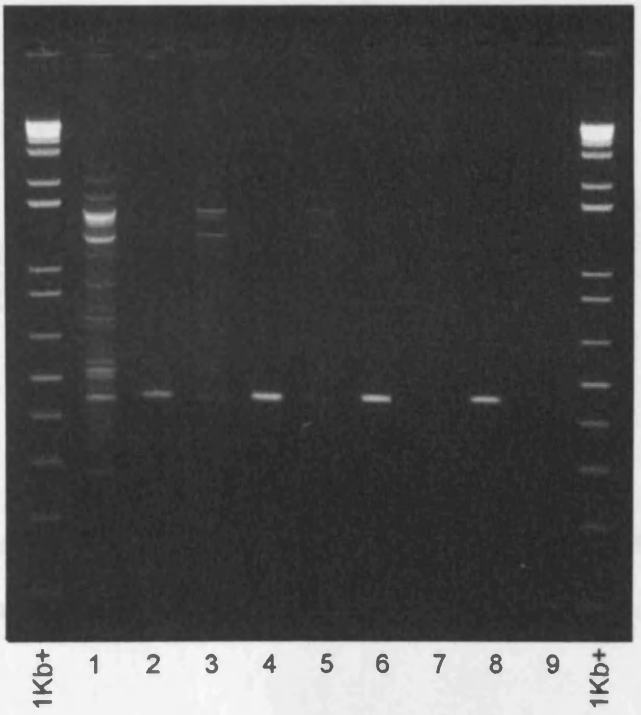


Figure 3-5. Serial dilutions of HPV-negative HaCaT DNA with adjacent signal from 1 in 40 SiHa DNA dilution for comparison. Ladder is 1kB+ ladder.

The amount of non-specific amplification decreased with decreasing concentration of HaCaT DNA and was minimal by 1 in 10 dilution (34ng target). Close inspection of the prominent band seen between 400 and 500 bp in lane 2 shows a clear separation in size between this band and the adjacent band from the HPV-positive SiHa DNA template. Southern blotting and probing with a HPV 16 probe was performed by Ms A. Nicholson. In contrast to the SiHa lanes, no signal from the HaCaT lanes was observed suggesting that the product did not originate from an HPV 16 template. In addition, a subsequent nested PCR with GP5+/6+ primers failed to produce any product from the HaCaT DNA (data not shown) supporting this amplicon as a non-specific PCR product.

3.2.1.6 MY09/11 primer system appears sensitive in detecting HPV 16 infection

A repeat of the sensitivity PCR described in section 3.2.1.2 was performed to allow comparison of the efficacy of MY09/11 and GP5+/6+ primer systems.

a)

		SiHa cells
Lane	*Dilution*	*Copies / 2µl*
1	Neat	3,400
2	1 in 10	340
3	1 in 30	113
4	1 in 40	85

b)

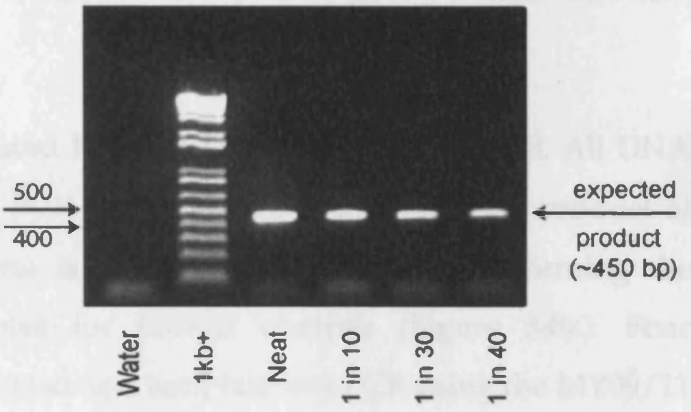


Figure 3-6. a) Concentration of SiHa template per 2µl PCR load b) MY09/11 primer PCR showing reducing product with increasing dilution. Product clearly visible at 85 copies HPV 16 per PCR.

The water control was appropriately negative. Serial dilutions of the SiHa cell line DNA showed a gradual decrease in the amount of PCR product (~450bp amplicon) from this primer system. Product was still readily visible at 85 copies

HPV 16 per PCR and the sensitivity for the detection of HPV 16 therefore appears comparable to the GP5+/6+ primer system.

3.2.1.7 Nested PCR increases sensitivity for the detection of HPV

Where sufficient DNA remained nested PCR was employed to assess those clinical samples initially negative for HPV infection by GP5+/6+ PCR (section 2.2.2.5.1). This technique relies on the positioning of the GP5+/6+ primer pair within the L1 amplicon generated by the MY09/11 primer system (Figure 3-7).

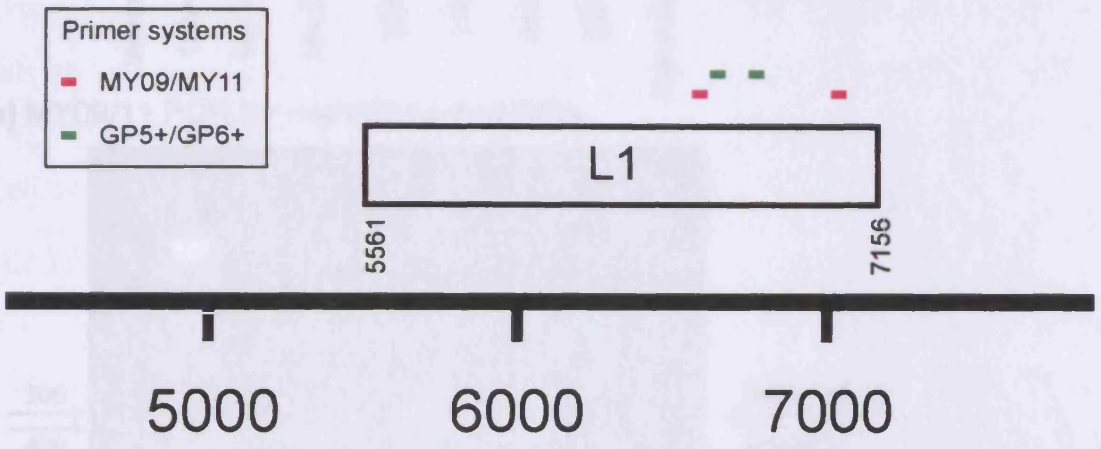


Figure 3-7. Relationship of MY09/MY11 (pink) and GP5+/GP6+ (green) primers to L1 ORF.

Sequence data shown for HPV 16.

Illustrative gels from such a nested PCR are shown in the figure 3-8. All DNA samples were first assessed by PCR for the β-globin gene. A clear product of expected amplicon size (250bps) is visible for each sample confirming the suitability of the DNA samples for further analysis (Figure 3-8a). Four microlitres of DNA extract was used as a template in a PCR using the MY09/11 primer system. A non-specific amplification pattern is seen for all of the clinical samples (Figure 3-8b; 238-293). Three negative controls were included for this experiment. They were 'no DNA/water' and two samples of the extraction buffer used to extract the clinical DNA samples (figure b; mix 1 and mix2). For the nested PCR reaction, 0.5µl of the primary MY09/11 PCR product was used as the PCR template in a GP5+/6+ primer PCR that was limited to thirty cycles.

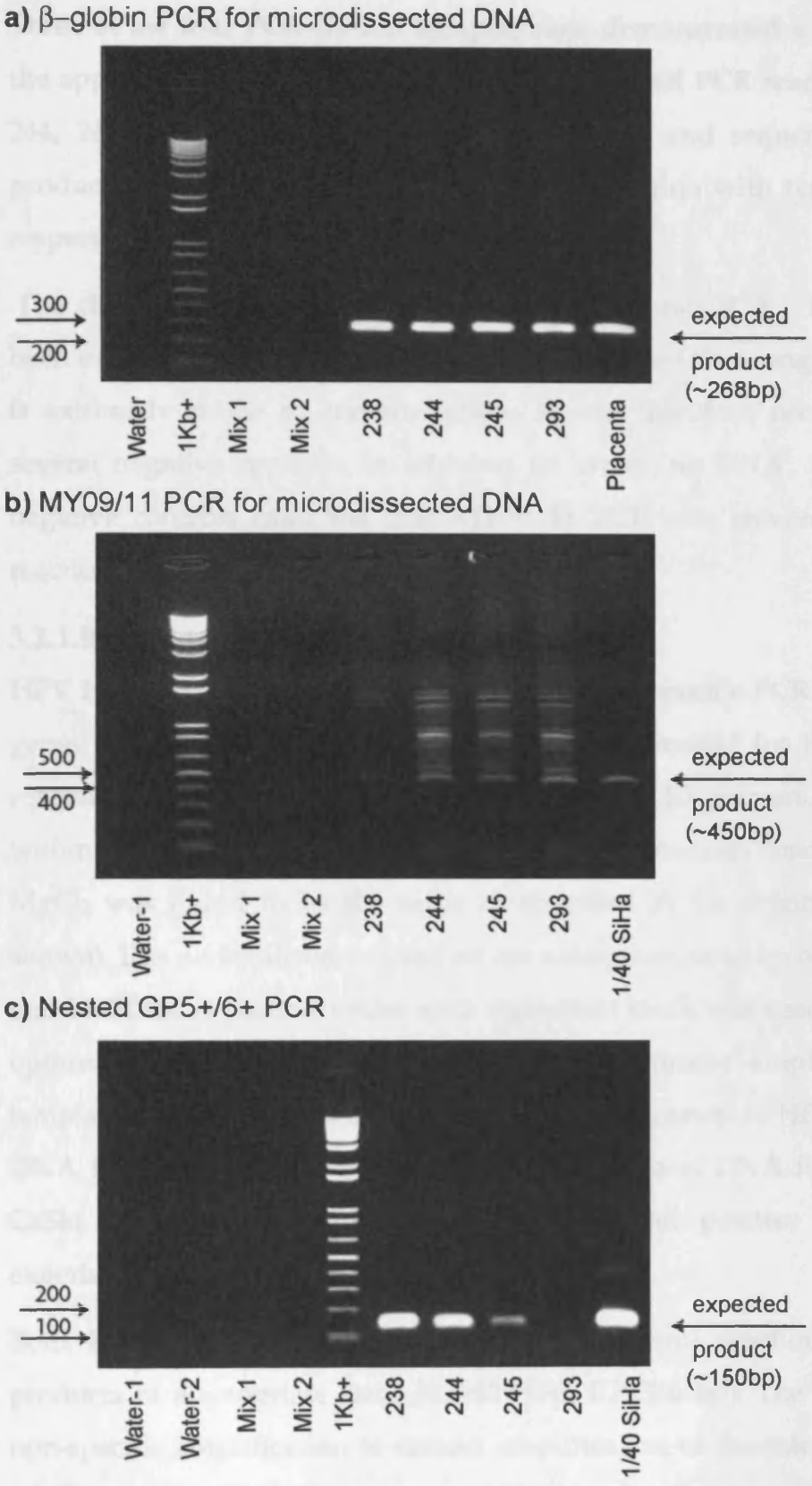


Figure 3-8. Three stages of nested PCR for HPV typing. a) Microdissected DNA assessed by β-globin PCR to ensure adequate quantity and quality of DNA preparation. **b)** Subsequent MY09/11 PCR shows non- specific type ladder for all clinical samples. **c)** Nested GP5+/6+ PCR using 0.5μl of MY09/11 product as a template. Clear product of the expected size visible for 238,244 and 245. Sample 293 remains negative. All negative controls were taken through both rounds of the nested reaction to ensure no contamination.

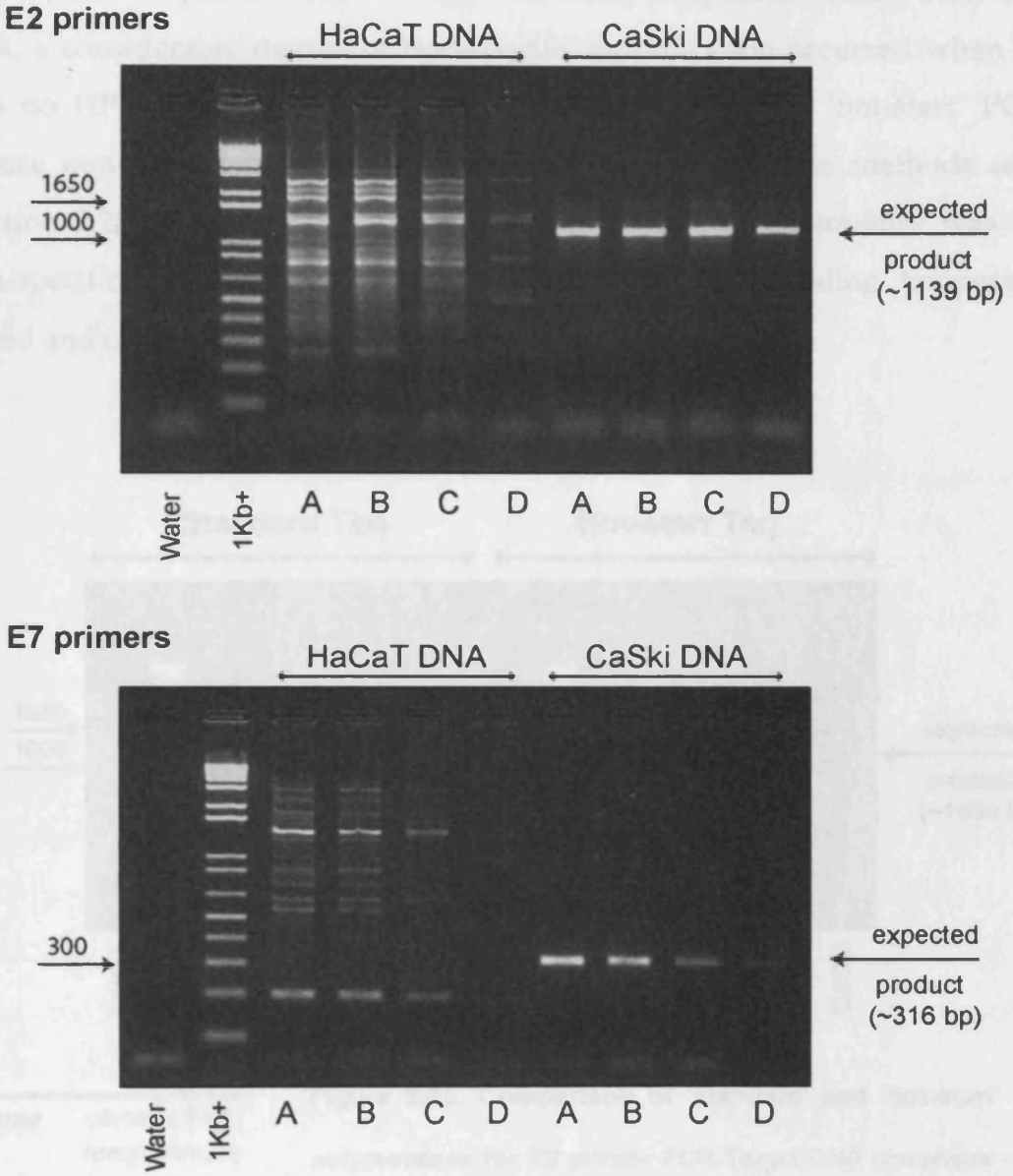
Three of the four PCR clinical samples now demonstrated a 'clean' product of the appropriate amplicon size following the nested PCR reaction (subjects 238, 244, 245; Figure 3-8c). Subsequent purification and sequencing of the PCR products from this reaction revealed HPV infection with types 16, 6b and 16 respectively.

The choice of negative controls is crucial to nested PCR. The technique has been estimated to be up to 10^4 times more sensitive than single round PCR and is extremely prone to contamination. It was therefore necessary to include several negative controls. In addition to 'water/no DNA', template from all negative controls from the first MY09/11 PCR was entered into the nested reaction (Figure 3-8c; lanes 1-4).

3.2.1.8 PCR to assess the physical state of HPV

HPV 16 positive samples were assessed by type specific PCR for the E2 and E7 genes (Das *et al.*, 1992). The reactions were optimised for the apparatus and reagents used in this thesis. An alternative set of E7 primers, already available within the group, was used. The optimum magnesium concentration (2.5mM $MgCl_2$ was found to be the same as specified in the original paper (gel not shown). Das and colleagues used an annealing temperature of 55°C for both E7 and E6 PCRs. A thermal cycler with a gradient block was used to determine the optimum annealing temperature to ensure efficient amplification of HPV template without unwanted non-specific amplification of HPV-negative DNA. DNA from HPV-negative HaCaT cells (300ng) and DNA form HPV-positive CaSki cells was used to provide negative and positive controls for this experiment (Figure 3-9).

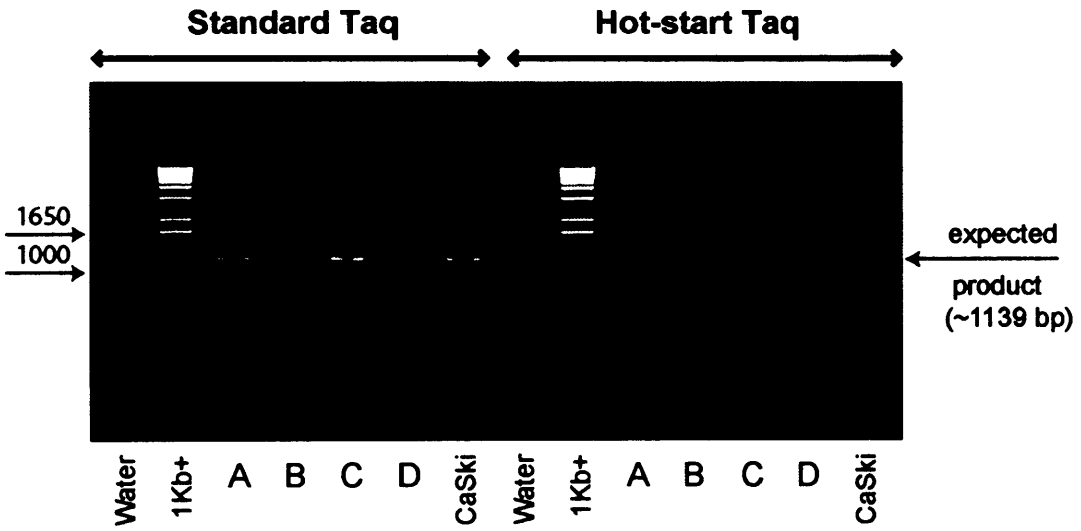
Both PCRs showed appropriate negative control reactions and produced products of appropriate sizes (E2=1139bp, E7=316 bp). The optimum ratio of non-specific amplification to correct amplification of the intended target DNA was achieved at 58°C E2 primers and 56°C for the E7 primers.



Lane	*Annealing temperature / °C*
A	55.1
B	55.9
C	57.8
D	60.9

Figure 3-9. Gradient PCR to establish the optimal annealing temperature for the E2 and E7 primer pairs used in the PCR to assess viral integration. HPV negative HaCaT DNA used as template to investigate non-specific amplification. Dilution of CaSki cell DNA (<150 copies of HPV 16) used to assess sensitivity of PCR. Optimal sensitivity with minimum non specific amplification appears to occur at ~58°C for the E2 primers and ~56°C for the E7 primers.

Initial optimisation of the E2/E7 PCR was performed using a cheaper Taq polymerase from Amersham Biosciences UK. It can be seen from Figure 3-9 (above) that despite the use of a high annealing temperature of 58°C for the E2 PCR, a considerable degree of non-specific amplification occurred when there was no HPV template (Figure 3-9; lanes 2-4). The use of 'hot-start' PCR to reduce non-specific amplification has been discussed in the methods section (section 2.2.2.1.4). When an appropriate hot-start Taq polymerase was used, non-specific amplification appeared reduced at all annealing temperatures tested and optimal at ~59°C (Figure 3-10, below).



Lane	*Annealing temperature / C*
A	57.5
B	58.2
C	58.7
D	59.0

Figure 3-10. Comparison of 'standard' and 'hot-start' Taq polymerases for E2 primer PCR. Target DNA comprises ~150 copies of HPV 16 extracted from CaSki cells mixed with 300ng of HPV-negative DNA from HaCaT cells. Reduced non-specific amplification visible with 'hot-start' Taq polymerase. Optimum sensitivity/specificity seen at 59°C.

3.2.2 Riboprobe synthesis

Viral gene expression was assessed using mRNA ISH. The synthesis of the riboprobes and precise localisation is described in the methods (section 2.2.3). The relationship of the riboprobes to the HPV 16 ORFs is shown in Figure 3-11.

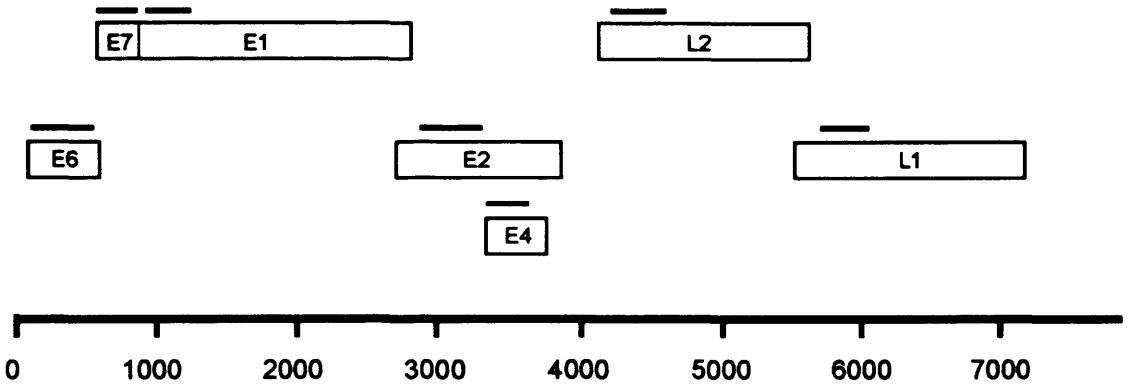


Figure 3-11. HPV16 riboprobes. HPV16 genome is shown in linear fashion. Boxes denote open reading frames. Green lines show positions of riboprobes.

All pGEM-3Z constructs were sequenced using either SP6 or T7 promoters to confirm the appropriate sequence had been cloned into the vector. Digoxigenin-UTP labelled riboprobes were generated using a proprietary digoxigenin-RNA labelling kit (Roche Diagnostics Ltd.). Dot-blot hybridisations using serial dilutions of probe were used to establish the relative concentrations of each probe (Figure 3-14). Concentrations were compared to a positive control provided by the manufacturer. Synthesis of anti-sense riboprobes using T7 RNA polymerase appeared more effective than that of sense riboprobes using SP6 RNA polymerase. Once synthesized, riboprobes were stored at -20°C until required.

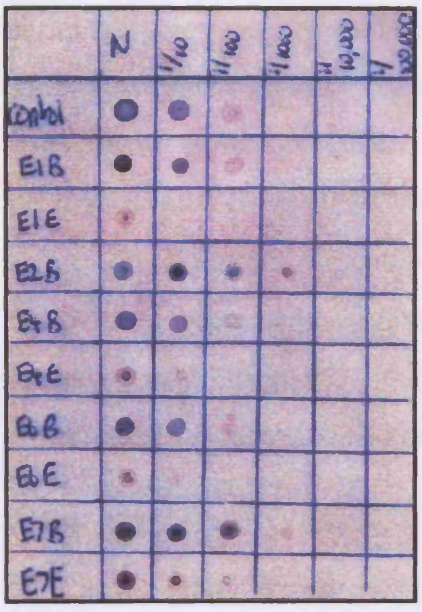


Figure 3-12. Dot-blot hybridisation of newly synthesized riboprobes. Each row represents different probe. 'B' signifies construct linearised with BamH1 and transcribed with T7 RNA polymerase to yield anti-sense RNA probe. 'E' signifies construct linearised with EcoR1 and transcribed with SP6 RNA polymerase to yield sense RNA probe. Anti-sense probes appear to be more efficiently synthesised than the sense probes and are detectable at greater dilutions.

3.2.3 Source of tissue samples

Fresh and paraffin embedded tissue samples were obtained from patients referred to the gynaecological oncology centre and specialist vulval clinics at Addenbrooke's NHS trust, Cambridge between April 1999 and May 2002. Prior ethical approval for this work was obtained from the local research ethics committee (LREC 98/227).

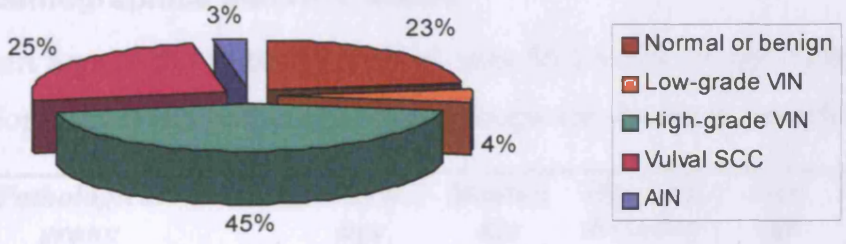


Figure 3-13. Pathology of vulval biopsies from sixty-seven individuals. (Six serial biopsies are included where the subsequent pathology differed from the index biopsy)

A total of one hundred and forty-eight specimens were available from sixty-seven individuals during the study period. Thirty-two individuals underwent

serial biopsies during their clinical management over the course of the study. The histopathological diagnosis was confirmed independently by two consultant gynaecological histopathologists. Where variation in opinion occurred, both consultants reviewed the case and reached an agreed diagnosis. Approximately half the biopsies obtained were classed as showing VIN and the majority of these were high-grade lesions. The sixteen benign lesions included histologically normal biopsies; biopsies showing HPV change only; inflammatory dermatoses and benign papilloma.

3.2.4 Statistical methods used in analysis

The small size of the cohort studied meant that non-parametric statistical methods were employed. Fishers exact test for the comparison of independent variables was used to compare proportions between two groups e.g. HPV status between groups. When the variable to be compared was continuous e.g. age, the Mann-Whitney U test was used. Data was compiled using Access 2000® and Excel 2000®. Statistical analysis was performed using SPSS 11.5®. Two-tailed tests were used and significance was assessed at the 95% level ($p < 0.05$).

3.3 Results

3.3.1 Demographics and HPV status

The mean age of the patients studied was 55.2 years (range 32-86 years). The details for each of the pathological subgroups are shown in the following table:

Pathological group	*N*	*Mean age (yrs)*	*Median age (yrs)*	*Standard deviation (yrs)*	*Min. age (yrs)*	*Max. age (yrs)*
Benign/normal	17	49.4	48.0	13.3	33	74
Low-grade VIN	3	42.3	43.0	1.2	41	43
High-grade VIN	33	50.8	48.0	10.6	32	78
Vulval SCC	18	70.5	71.5	10.8	49	86
AIN	2	50.0	50.0	4.24	47	53

Table 3-2. Age distribution by pathological subgroup

The median age for the patients with VIN was 48 years (range 32-78 years) compared to 72 years (range 49-86) for those with SCC of the vulva ($p<0.001$). The majority (72.6%) of patients studied were HPV-positive, including 94.3% of those patients with VIN. Subjects with VIN were significantly more likely to have HPV infection than those with carcinoma (94.3% vs. 58.8%; $p=0.003$). The predominant infective agent in cases of VIN was HPV 16 (93.9%) with types 33 and 56 accounting for the remaining cases. PCR data regarding the physical state of the virus was available in 24/31 HPV 16 positive cases. HPV 16 was found to be integrated in half the cases of HPV 16-positive VIN. Serial biopsies over at least six months were available for longitudinal assessment of HPV status in fifteen patients. HPV status and the predominant HPV type isolated by PCR remained stable in 11/15 (73.3%) of cases. Of the remaining four cases, two became HPV negative following the successful treatment of their disease; one showed resolution of HPV infection without resolution of the disease and one patient showed a varying type between type 16 and type 33.

The majority of the thirty-six women with VIN were symptomatic (87.8%; data unavailable in 3/36 cases). The most common symptoms were itch (60.0%) and soreness (36.7%). Other reported symptoms included the finding of a lump (20.0%), discoloration (13.3%) or more rarely bleeding or discharge (6.7%). Accurate data on the duration of symptoms was available in 27/37 women with VIN. The median duration of symptoms was 24 months (range 3-180 months). Almost two-thirds (62.2%) of patients with VIN had already undergone previous treatment at the time of their referral. Although more women with VIN were smokers than those with benign vulval pathology, this difference was not statistically significant (45.7% vs. 33.3%; $p=0.537$). The current cervical smear was abnormal in 12.5% of VIN patients, but almost half (48.6%) had a history of abnormality on previous cervical cytology.

3.3.2 *In situ* hybridisation – tissue samples and casemix

RNA *in situ* hybridisation was performed on paraffin-embedded sections. Prior ethical approval for this work was obtained from the local research ethics

committee (LREC 98/227). Sense probes were used on paired sections as a negative control for the hybridisation reaction. Experiments were repeated to ensure reproducibility. Sixty-three biopsies from thirty-one individuals were assessed during the study. The majority of samples taken were from high-grade VIN lesions, but specimens from vulval SCCs, high-grade AIN and both normal and inflammatory HPV-positive vulval skin were also available for assessment. Biopsies from several time points (range 2-5) were available from fourteen individuals. In nine of these patients the multiple biopsies demonstrated vulval intraepithelial lesions of an identical histopathological grade, whilst in five cases the grade of lesion varied between time points.

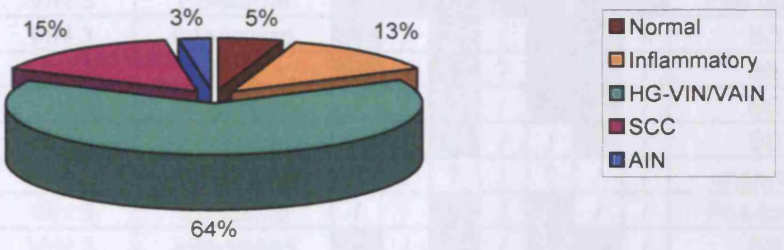


Figure 3-14. Relative frequency of pathological diagnoses from thirty-one individuals assessed by RNA *in situ* hybridisation

3.3.3 *In situ* hybridisation - results

Following RNA *in situ* hybridisation, sections were mounted under glass coverslips and assessed by conventional light microscopy by two observers (the author and Dr. J. Sterling). The results are detailed in Table 3-3 (overleaf).

Sample No.	Primers for HPV typing	Diagnosis	Physical state of virus	HPV 16 riboprobe							RNA summary
				E1	E2	E4	E6	E7	L1	L2	
208	GP	Normal	Integrated	+	/	+	/	+	+	/	BS
224	GP	Normal	N/A	+	/	+	-	/	+	/	BS
266	TS	Inflam	Episomal	/	-	-	/	-	-	/	Neg
268	TS	Inflam	Episomal	/	/	+	/	+	/	/	BS
278	GP	Inflam	Integrated	/	/	?	/	+	/	/	Neg/BS
277	TS	Inflam	Integrated	/	/	+	/	?	/	/	Bs
256	N	Inflam	Episomal	/	/	+	/	+	/	/	BS
262	N	AIN 3	Episomal	/	/	+	/	-	/	/	BS
210	GP	VAIN 2	Integrated	+	+	+	+	+	+	/	Pos-lower
251	GP	VIN 2	Episomal	/	/	+	?	+	/	/	BS
282	GP	VIN 2	Episomal	/	-	-	/	+	-	/	BS
241	GP	VIN 2	Integrated	-	/	+	/	+	+	/	BS
265	GP	VIN 3	Episomal	?	-	+	+	+	+	/	Pos-lower
220	GP	VIN 3	Integrated	/	+	?	/	+	+	/	BS
239	GP	VIN 3	Integrated	/	+	+	/	+	+	+	BS
253	GP	VIN 3	Integrated	+	/	+	/	+	+	/	BS
223	GP	VIN 3	Episomal	+	?	+	+	+	+	+	Pos-upper
250	GP	VIN 3	Episomal	+	+	+	+	+	+	+	Pos-upper
252	N (56)/TS	VIN 3	Integrated	+	/	+	/	+	+	/	BS
260	N (56)	VIN 3	N/A	+	/	+	?	+	-	/	BS
272	TS	VIN 3	Integrated	/	/	+	/	+	+	/	BS
273	GP	VIN 3	Episomal	/	/	+	/	/	/	/	BS
305	N	VIN 3	Episomal	/	/	?	/	?	/	/	uncertain
283	GP	VIN 3	Episomal	/	/	+	/	+	/	/	Pos-lower
205	GP	VIN 3	Integrated	+	/	+	/	+	+	/	BS
230	GP	VIN 3	N/A	/	+	-	/	+	+	/	bs
233	GP	VIN 3	Integrated	+	+	+	/	+	+	/	BS,?Pos-lower
209	GP	VIN 3	N/A	+	/	+	/	-	+	/	BS
199	GP	VIN 3	Integrated	/	?	+	/	+	+	/	BS
215	GP	VIN 3	Integrated	/	+	+	/	+	+	/	bs
226	GP	VIN 3	N/A	/	+	+	/	+	+	/	BS
243	N	VIN 3	Integrated	/	/	+	/	-	/	/	bs
257	GP	VIN 3	Episomal	/	/	+	/	+	/	/	BS
267	GP	VIN 3	Episomal	/	/	+	/	/	/	/	BS
225	N	VIN 3	Episomal	/	+	+	/	+	+	/	BS
204	GP	VIN 3	Episomal	+	+	+	+	+	+	+	Pos-upper
217	GP	VIN 3	Episomal	+	+	+	+	+	+	?	Pos-upper
234	GP	VIN 3	Episomal	+	+	+	+	+	+	+	Pos-upper
275	GP	VIN 3	N/A	+	+	+	+	+	+	/	Pos-upper
306	MY/ GP	VIN 3	Episomal	/	/	+	/	/	/	/	Pos-upper
219	GP/TS	VIN 3	Negative	/	+	+	/	/	?	/	BS
221	GP	VIN 3	Integrated	/	/	/	/	+	?	+	bs
242	GP	VIN 3	Integrated	/	/	+	/	?	/	/	bs
255	GP	VIN 3	Integrated	/	/	?	+	-	/	/	Neg/bs
202	GP	VIN 3	Episomal	+	+	+	+	+	+	+	Pos-upper

Sample No.	Primers for HPV typing	Diagnosis	Physical state of virus	HPV 16 riboprobe							RNA summary
				E1	E2	E4	E6	E7	L1	L2	
216	GP (33)	VIN 3	N/A	+	+	+	+	/	+	+	Pos-upper
235	GP	VIN 3	Episomal	/	/	+	+	+	/	/	Pos-upper
248	N	VIN 3	Episomal	+	+	+	+	+	+	/	Pos-upper
264	GP	VIN 3	Episomal	/	/	+	/	+	/	/	BS
211	GP	VIN 3	Integrated	-	+	+	/	+	-	/	BS
222	GP	VIN 3	Integrated	/	-	-	/	+	?	/	bs
254	GP	VIN 3	Integrated	+	/	+	/	+	+	/	BS
228	GP	VIN 3	Episomal	+	+	+	+	+	+	+	Pos-upper
214	GP	VIN 3	Integrated	+	/	-	/	?	?	/	uncertain
232	GP	VIN 3	Integrated	/	?	+	/	?	?	/	uncertain
245	GP	VIN 3	Integrated	/	-	-	/	?	/	/	Neg
304	TS	VIN 3	Episomal	/	/	+	/	+	/	/	BS
249	MY	SCC*	Episomal	/	/	+	/	+	/	/	Pos-upper
284	GP/N (0)	SCC	Negative	/	/	-	/	?	/	/	uncertain
274	N	SCC	Episomal	/	/	-	/	/	-	/	Neg
238	N	SCC	Negative	/	/	?	/	?	/	/	uncertain
263	MY/N	SCC	Integrated	/	/	+	/	+	/	/	bs
259	TS	SCC	Integrated	/	/	+	/	+	/	/	BS

Table 3-3. Overview of RNA in situ hybridisation with digoxigenin labelled riboprobes. Specimen numbers in italics indicate those subjects from whom multiple biopsies were assessed. Primers used for HPV typing indicated – 'MY'– MY09/11; 'GP' – GP5+/6+; 'N' – nested PCR and 'TS' – type-specific primers. Where typing PCR indicated presence of different HPV type, this indicated in brackets. 'Inflam' indicates the histological diagnosis of inflammatory dermatoses. Presence of signal observed with riboprobes tested is indicated by '+' and lack of a signal by '-'. '/' Indicates that the probe was not used to test that specimen. The predominant RNA pattern observed for all probes used is specified in right hand column. 'Pos' - positive; 'Neg' – negative and 'BS' – blush and speckle pattern. 'bs' indicates a weaker signal than 'BS'. This classification is based on assessment by two observers. Where consensus could not be reached, the pattern is specified as 'uncertain'.

*specimen classed as SCC, but gene expression appeared to be within adjacent VIN.

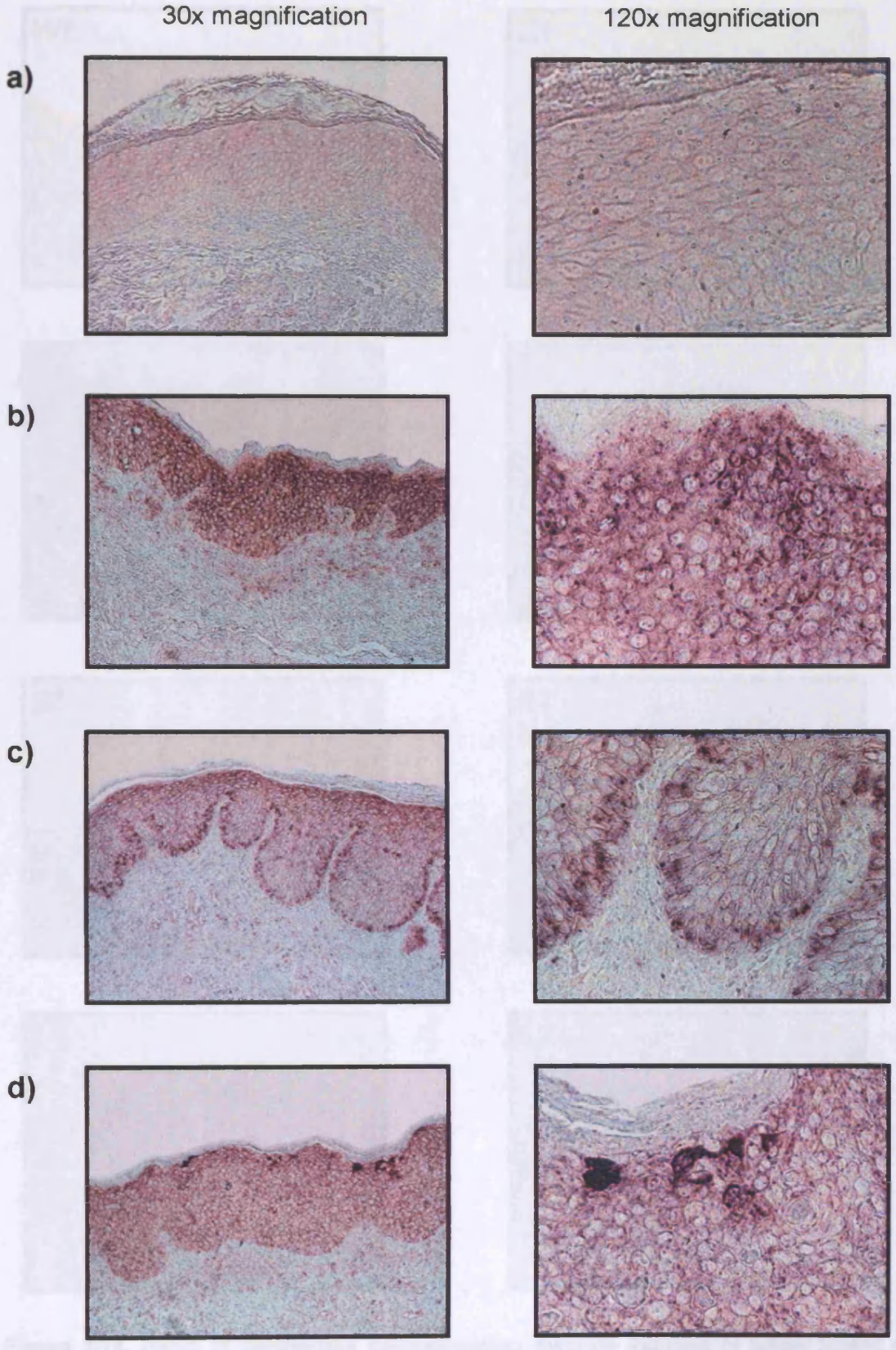


Figure 3-15. Major patterns of viral transcription identified by RNA *in situ* hybridisation. All images are following ISH with HPV16 E7 riboprobes. **a)** Negative pattern; **b)** Blush-speckle pattern; **c)** Positive-lower pattern and **d)** Positive-upper pattern. Left hand images at 30x magnification. Right hand images at 120x magnification. Positive staining patterns taken from cases of VIN 3. Negative images from case of inflammatory dermatosis.

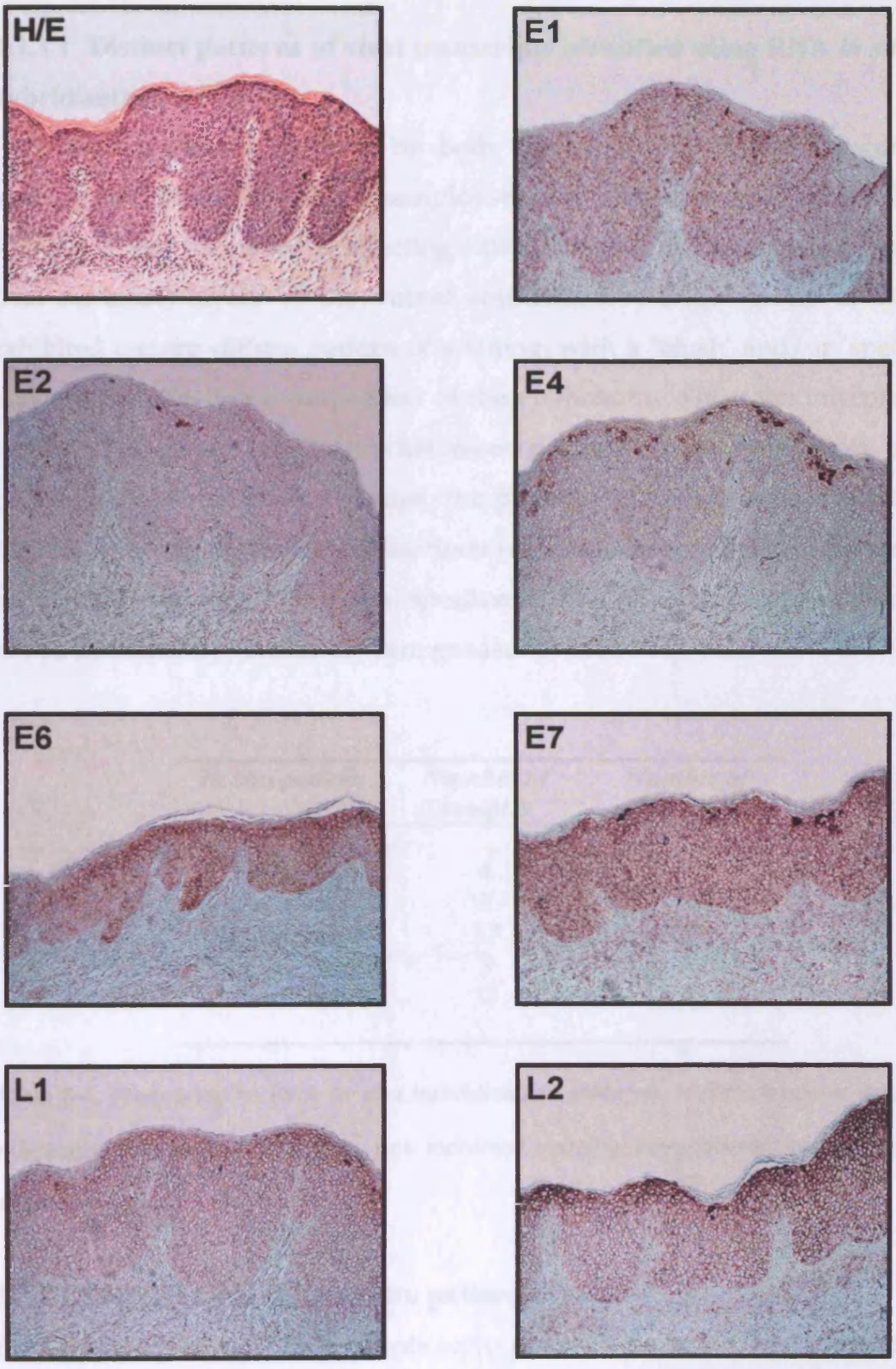


Figure 3-16. Panel of riboprobes demonstrating discrete staining of upper layers of epithelium. All images at 30x magnification. H/E section shows characteristic appearance of undifferentiated VIN3 (case 202). Strongest RNA ISH signal typically obtained with riboprobes for HPV 16 E4 and E7.In this case, a discrete pattern of staining affecting upper epithelium is clearly visible. L2 riboprobe signal exhibits 'blush' pattern of positive staining.

3.3.3.1 Distinct patterns of viral transcripts identified using RNA *in situ* hybridisation

For samples deemed positive by both assessors, three broad categories of staining were identified. Some samples showed discrete nuclear or cytoplasmic staining clearly identified as affecting either the upper (more common) or lower (less common) layers of the vulval epithelium. A larger group of samples exhibited a more diffuse pattern of staining, with a 'blush' and/or 'speckling' appearance, affecting a proportion of the epithelium. Whilst the interpretation of this third group was somewhat more subjective than the distinct pattern observed for the other two groups, the pattern did appear to be distinct from both the negative control hybridisations with the sense probes and the negative results observed for some clinical specimens. The results for biopsies that could not be classified by consensus were graded 'uncertain'.

In situ pattern	*Number of samples*	*Number of individuals*
Negative	4	4
Uncertain	5	4
Blush/Speckle	37	22
Positive - lower	3	3
Positive - upper	13	5

Table 3-4. Frequency of RNA *in situ* hybridisation patterns. Multiple biopsies from seven individuals were graded differently, one individual showing three different patterns for three separate biopsies.

3.3.3.2 Variations in RNA *in situ* pattern over time

Biopsies from multiple time points were available from fourteen patients, nine demonstrating the same grade of intraepithelial neoplasia and five patients in whom the histological grade varied by one level. For patients with a consistent histological diagnosis the RNA *in situ* pattern also remained consistent. The pattern of staining however differed between cases. Of the remaining five

patients in whom the histological diagnosis varied between biopsies, three patients also showed a variation in the predominant pattern of mRNA observed by *in situ* hybridisation. For two of these patients, an increased histological grade was associated with the finding of a discrete as opposed to diffuse mRNA pattern of *in situ* staining (sample numbers 251/265 and 224/210).

3.3.3.3 The effect of immunotherapy on viral transcription as assessed by RNA *in situ* hybridisation

Twelve patients who provided specimens for this work were subjects participating in a vaccination trial that will be described in detail later in this thesis (Chapter 5). These patients were vaccinated with a viral vector vaccine encoding the high-risk oncoproteins E6 and E7 from HPV type 16 and 18. Subjects were followed over a six-month period with respect to the development of HPV-specific cytotoxic immunity and potential clinical effect. Ten of the patients did not show a change in the pattern of RNA *in situ* hybridisation demonstrated following vaccination. For the two remaining patients, whilst the predominant pattern observed did not change, differences were observed for the high-risk oncoproteins. In one patient, who acheived complete clearance of her high-grade VAIN, mRNA for HPV 16 E6 was detectable at screening but undetectable 3 months following vaccination when her disease had resolved (patient YF, samples 210 and 224). In the second of these patients, HPV 16 E7 mRNA was evident on the pre-vaccination biopsy, but not on the biopsy taken at six months following vaccination (patient FS, samples 221 and 255). This patient had partial regression of her disease.

3.4 Discussion

The patients studied in this chapter appear typical of those with VIN and vulval SCC. The median age of 48.0 yrs for patients with high-grade VIN is similar to both institutional and population data reported previously (Buscema *et al.*, 1988; Sturgeon *et al.*, 1992; Jones *et al.*, 1994; Haefner *et al.*, 1995; I.S.G.V.D., 1996; Iversen *et al.*, 1998; Joura *et al.*, 2000). Over two-thirds of patients with VIN were

under the age of fifty, lending support to the high-incidence of the disease previously reported in younger women (Jones *et al.*, 1994; Herod *et al.*, 1996; Joura *et al.*, 2000). As might be expected, patients with invasive disease were significantly older than those with VIN.

The vast majority of women with VIN in the present study were symptomatic, a finding in agreement with many previous studies (see section 1.1.5). The most common symptoms were those of pruritus and soreness. The high median duration of symptoms (24 months), as well as the fact that almost two-thirds of subjects had undergone previous treatment for their VIN, confirm the chronic, often recurrent nature of this condition. A history of current or previous abnormal cervical cytology in almost half our cohort of patients with VIN is in keeping with the findings of an earlier British study (Herod *et al.*, 1996). However, the incidence of smoking in the cohort with VIN (45.7%) was perhaps not as high as has been previously described (Jones *et al.*, 1994; Modesitt *et al.*, 1998).

The results presented in this chapter confirm earlier suggestions of the strong association between high-grade VIN and HPV infection (Haefner *et al.*, 1995; Hørding *et al.*, 1995; van Beurden *et al.*, 1995; Madeleine *et al.*, 1997; van Beurden *et al.*, 1998a). The strategy of testing with MY09/11 and GP5+/6+ primer systems both independently and in a nested fashion should detect HPV with high sensitivity and the high incidence of HPV infection in patients with VIN (~95%) would support this. It should be noted that the data presented in this chapter only applies to basaloid/warty VIN. Differentiated VIN was not studied in the current work. The data would tend to support the views of Hørding and colleagues, who feel that VIN 3 is indicative of underlying HPV infection (Hørding *et al.*, 1993). In keeping with previous studies (reviewed in section 1.1.4.1) HPV infection was significantly more common in those patients with VIN as opposed to SCC of the vulva (94.3% vs. 58.8%; p=0.003). The low number of patients with SCC of the vulva makes it hard to draw comparisons between HPV-positive and HPV-negative individuals. It was not possible to classify the SCCs further into basaloid/warty and keratinising subtypes.

The GP5+/6+ primer system was initially chosen instead of the MY09/11 system to take advantage of the fact that those primer systems that amplify smaller products are typically more sensitive in detecting the desired target DNA. PCR to identify genital HPV infection was not available within the research group and it was therefore appropriate to ensure optimisation of one PCR system in the first instance. With increasing expertise in the technique of PCR, the MY09/11 primer system was optimised and used to test samples that were negative for HPV by GP5+/6+ PCR. The GP5+/6+ and MY09/11 primer systems vary in their ability to detect different genital HPV types (Qu *et al.*, 1997) and using both to screen for HPV infection could therefore increase the ability to detect HPV within a clinical sample. Qu and colleagues found the GP5+/6+ and MY09/11 systems to be equally effective with regard to the detection of HPV 16, the most common viral type isolated from patients in this study. However, some samples were found to be positive for HPV 16 by MY09/11 rather than GP5+/6+ PCR and it would therefore appear that the use of both primer systems might increase the sensitivity of HPV detection.

As expected, nested PCR for HPV detection was more sensitive than single-round PCR. It could therefore be argued that the nested PCR typing system should have been employed from the start of this project. However, the additional sensitivity occurred at the expense of a high risk of contamination. The negative controls (water and extraction mix) must be taken through both rounds of PCR for the result to be valid. Whilst a high sensitivity for the detection of HPV infection is helpful in ensuring cases labelled as 'HPV-negative' are truly negative, the biological relevance of cases designated as 'HPV-positive' on the basis of a highly sensitive PCR, able to detect HPV at limited copy number, may be questionable. From a clinical viewpoint, the demonstration of viral transcription or integration may have more of an impact on the pathogenesis of neoplasia than HPV infection alone. However, the limited data available suggests that infection in normal vulval skin is infrequent. Using type-specific PCR, Hørding *et al.* failed to demonstrate HPV in 101 normal vulval biopsies (Hørding *et al.*, 1993). This finding is in stark

contrast to the uterine cervix, where transient infection with HPV in the absence of dysplasia is a common occurrence (Ho *et al.*, 1998). The different architecture of the transformation zone may account for the ease with which HPV can access the cervix but as yet unidentified difference in the host immune response may also play a part in the variation in HPV infection with anatomical site. Vulval HPV infection may be more significant than its cervical counterpart and it is therefore appropriate to use a highly sensitive PCR technique when assessing vulval specimens.

The high incidence of HPV 16 infection in VIN is in keeping with the findings of others (discussed in detail in section 1.1.4.1). Direct sequencing has been used to demonstrate frequent HPV 16 infection in VIN (van Beurden *et al.*, 1995; Rosenthal *et al.*, 2001). A potential limitation of direct sequencing for HPV typing is the inability of the method to detect multiple infections. The use of a second round of type-specific PCR (van den Brule *et al.*, 1990) or hybridisation of the PCR product to type-specific probes (Kleter *et al.*, 1999) could have overcome this problem. However, previous studies that have used methodology able to detect multiple HPV infection have shown that such infections are unusual in VIN (Haefner *et al.*, 1995; Hørding *et al.*, 1995; Madeleine *et al.*, 1997). This fact, coupled with the available resources and timescale of the current project, led to the choice of direct sequencing to type clinical specimens. Three samples from two individuals produced a positive signal following HPV 16 RNA *in situ* hybridisation despite the isolation of a different HPV type by PCR/sequencing (samples 216, 252 and 250 - HPV type 33, 56 and 56 respectively). In one of these samples (sample 252), the prescence of HPV 16 was confirmed using type-specific PCR, indicating that the RNA ISH result reflected infection with multiple HPV types rather than non-specific hybridisation of the HPV 16 riboprobe. It would therefore appear that, although uncommon, infection with multiple HPV types can occur in VIN. The MY09/11 primer pair system may be more effective in amplifying multiple HPV types from a single specimen than its GP5+/6+ counterpart (Qu *et al.*,

1997) and may therefore be the most appropriate primer system should information on multiple infections be required.

The serial nature of biopsies in fifteen individuals provides some information as to the stability of HR-HPV infection in individuals with high-grade VIN. In the majority (11/15) of these patients the predominant HPV type isolated following PCR remained the same. In one individual the type varied between HPV 16 and HPV 33, both recognised HPV types associated with high-grade VIN. It seems likely that this latter case may reflect a mixed infection, with variance in the HPV type amplified by PCR at differing time points. Three HPV-positive individuals became HPV-negative during follow-up. None of these can be regarded as spontaneous clearance of the virus as all had been treated either by vaccination with a viral vector vaccine directed against HPV types 16 and 18 (see Chapter 5) or with Imiquimod (a topical cytokine mediator described in section 1.1.7.3.3). In two of these patients resolution of the disease was accompanied by the disappearance of HPV from clinical specimens. The small numbers of patients with serial biopsies make it difficult to draw any firm conclusions from this dataset. As might be expected, it appears that most women with persistent disease remain HPV-positive and that the predominant type of virus does not change. This finding would support the role of HPV infection in the development of high-grade VIN.

Whilst no data exists for its role in VIN, viral integration has been proposed as a key step in the development of HPV-related carcinoma of the uterine cervix (section 1.2.1.5). Estimates of the percentage of high-grade CIN lesions containing integrated viral DNA vary considerably, but analysis of invasive disease has shown the vast majority of invasive lesions to contain integrated high-risk viral DNA (Park *et al.*, 1997; Kalantari *et al.*, 2001). Viral integration was assessed using PCR for the E2 gene as a surrogate marker, as this gene is commonly disrupted during integration into the host genome (Choo *et al.*, 1987; Das *et al.*, 1992). Approximately half the HPV-positive VIN samples available for analysis showed loss of this gene and were therefore designated as integrated. This finding would suggest that integration commonly occurs

before the development of malignancy and may serve to drive malignant progression in vulval neoplasia. Recent work on the W12 cervical cancer cell line would support the role of integration early on in oncogenesis. Integration in this cell line precedes the development of high-level chromosomal instability (Pett *et al.*, 2004). The authors found that increasing levels of the E7 oncoprotein correlated with increasing numerical and structural chromosomal aberrations. They postulate that loss of E2-mediated transcriptional repression lies behind increasing E7 protein levels and suggest that integration is a critical step in cervical carcinogenesis. Using an E2-based PCR to assess integration may have some problems. Loss of the E2 amplicon with a positive PCR for E7 is assumed to indicate disruption of the E2 ORF occurring during integration. The technique relies upon primer sequences located at either end of the E2 ORF, giving an amplicon of over 1kb in size. The amplicons from the HPV E7 ORF are typically much smaller (~300 kb). As such, loss of an E2 signal might simply reflect less effective amplification of the larger DNA target. This system may also underestimate the true extent of integration, as it does not allow the detection of mixed populations of cells where the virus exists in both episomal and integrant forms. Such cases will produce amplicons in both E2 and E7 PCRs and would therefore be classified as episomal by this technique. Some workers have proposed that the E2:E7 ratios provided by quantitative PCR may allow for discrimination of such mixed cell populations (Peitsaro *et al.*, 2002). The ratio thresholds of E2:E7 proposed in the study report by Peitsaro (1.0 ± 0.05 for episomal HPV) were however arbitrary and had not been statistically validated[3].

Other techniques are available to assess the physical state of HPV in clinical specimens. Restriction enzyme digestion (Matsukura *et al.*, 1989) can allow the distinction of episome from integrant by the pattern of bands observed following digestion with no-cut and single-cut restriction enzymes. This technique requires large amounts of DNA and would not have been suitable for this study. The recent development of sequence-based techniques may provide

[3] Professor S. Syrjänen, personal communication, HPV 2002, Paris.

more biologically relevant information regarding viral integration. The APOT technique (Amplification of Papillomavirus Transcripts) is an RT-PCR assay that allows the size-based distinction of mRNA transcripts derived form integrated viral DNA from those derived from episomes (Klaes *et al.*, 1999). Reverse transcription of all mRNA is initiated using an oligo$(dT)_{17}$ primer coupled to a linker sequence. A nested PCR using E7-specific primers and primers for the linker sequence then amplifies all viral E7 transcripts. The most common E6/E7 transcript from viral episomes is spliced from the E1 splice donor signal to the E4 splice acceptor site before termination at the polyadenylation site (Schwarz *et al.*, 1985; Sherman *et al.*, 1992). In contrast, transcripts from integrated DNA are longer and usually have viral sequences at their 5′ ends with flanking cellular sequences at their 3′ ends. APOT has the advantage that the technique identifies transcriptionally active DNA, which is likely to be of greater biological significance. Technical difficulties, mainly due to the limited stability of mRNA in clinical samples, limit the application of this technique. Fresh tissue samples were available for the majority of cases in the current work and this technique might have been employed.

The riboprobes synthesized in this study were able to detect RNA transcription within a subset of the vulval lesions studied. The E4 and E7 probes gave the strongest signals in positive cases (Figure 3-16). Initial experiments suggested that these probes appeared predictive of the likelihood of further positive signals with other riboprobes. In view of the limited availability of tissue from the small biopsies available, the initial *in situ* experiments were therefore performed with this pair of probes. The clarity and intensity of signal achieved with the E6 riboprobe was disappointing throughout the study. Whilst this may reflect low levels of E6 transcription, other authors have found transcripts initiated within E6 to be common within high-grade lower genital tract lesions of the vulva and cervix (Crum *et al.*, 1989; Park *et al.*, 1991b; Nakagawa *et al.*, 2000b). It may therefore be that the design or sequence of the probe was flawed. However, all pGEM-3Z constructs were sequenced prior to their usage, and each riboprobe was designed to fall within the relevant ORF using data from

the Los Alamos national laboratory HPV sequence library (http://hpv-web.lanl.gov/stdgen/virus/hpv/).

Three distinct patterns were seen for those HPV 16 cases found to be positive for RNA expression by *in situ* hybridisation with the riboprobes described earlier (Figure 3-15). The most prevalent pattern was that of 'blush' and 'speckle'. This pattern was sometimes observed throughout the entire thickness of the epithelium, but was limited to the upper one to two thirds in other cases. The widespread distribution of such staining raises the possibility that the pattern seen represented background signal rather than target mRNA expression. However, the corresponding negative signal seen with the sense probe for these cases would tend to suggest the result is valid. Indeed, Park *et al.* (1991b) used tritium-labelled sub-genomic probes to demonstrate transcripts throughout the full thickness of the epithelium in basaloid VIN 3. The authors found the topographical pattern of transcription for E6-E7 to be similar to that of L1-L2. In the current work, a similar relationship between the early and late transcripts was seen for those subjects with a 'blush-speckle' pattern. This wide distribution of transcripts is biologically plausible. If HPV is causally related to vulval neoplasia, one would expect transcription and translation to extend upwards from the area of viral entry in the basal epithelium. Furthermore, following evidence of the clonal nature of high-grade VIN (Tate *et al.*, 1997; Lin *et al.*, 1998; Pinto *et al.*, 2000), viral activity would be expected in all cells following clonal expansion. Such a diffuse distribution of transcripts does not seem to be unique to intraepithelial neoplasia of the vulva. A study of high-grade CIN also demonstrated early transcripts throughout the full thickness of the epithelium (Dürst *et al.*, 1992). It is important to note that the 'blush-speckle' pattern was also identified in biopsies from both histologically normal and inflammatory lesions. This finding is in contrast to that of Hørding *et al.* (1993) who found were unable to demonstrate HPV infection using PCR in over one hundred normal vulval biopsies. The significance of this infection is uncertain. Whilst transient HPV infection is extremely common in the cervix (Ho *et al.*, 1998) little is known about the natural history of infection in the

vulval area. HPV may in fact be active as a driving force for malignant change at an early stage, before the onset of histological change. Furthermore, the data presented here would tend to support those who feel that far from being mutually exclusive pathways, inflammatory dermatoses and HPV infection can act simultaneously as cofactors in the development of intraepithelial disease and malignancy (Kiene *et al.*, 1991; Ansink *et al.*, 1994; Haefner *et al.*, 1995). The data may however be biased. Biopsies of normal and inflammatory tissue were not collected in a truly prospective fashion and may not be representative of such lesions as a whole. Clinical practice dictates that painful biopsies are not routinely performed when there is little concern regarding the possibility of malignancy. Many subjects with no previous history of VIN or carcinoma will first undergo a period of observation or a therapeutic trial with topical agents such as steroids prior to their referral to the hospital clinic for assessment. As such, cases reviewed in the clinic will commonly be those with longstanding or resistant disease and those with a recent history of topical immunosuppressive use. Both these features may potentially increase the likelihood of HPV infection. Other biopsies were obtained from symptomatic patients or those attending colposcopy clinics with a history of cervical or vulval intraepithelial disease. These lesions may not therefore be truly reflective of normal vulval tissue or indeed inflammatory dermatoses.

Two patterns of discrete positive staining using the antisense riboprobes were seen in VIN, namely 'upper' and 'lower' patterns. In such cases, the staining pattern was readily identified and often focal in nature. Positive staining affecting the lower epithelium was demonstrated using the riboprobes for one of the high-risk transcripts, E6 or E7 (see Figure 3-15c). Other transcripts identified in these cases localised to the upper epithelium. The 'positive-lower' pattern would appear to fit most comfortably with the classical concept of subversion of cell growth in the basal cells of the epithelium as a result of HR-HPV infection. In productive infections, expression of E4 and the late genes occurs in the upper layers in a differentiation-dependent fashion (section 1.2.1.3). Recent work suggests that in cervical HSILs, the order of life cycle

events is preserved but the infection may be abortive with E4 and L1 often limited to small areas at the epithelial surface (Middleton *et al.*, 2003). Whilst late gene transcripts were never found in the lower layers of the epithelium, the limited number of cases exhibiting the 'positive-lower' pattern and restricted numbers of sections available for each case make it difficult to draw such conclusions with respect to the viral life cycle in VIN.

In one case of high grade VAIN exhibiting the 'positive-lower' pattern (case 210), a wide variety of transcripts were detected. These included the transcript for HPV 16 E2. This is of particular interest in view of the fact that the sample was thought to contain HPV 16 in its integrated form. As integration commonly occurs within the E2 region (Baker *et al.*, 1987; Schneider-Maunoury *et al.*, 1987), one would expect the E2 transcript to be missing in such a case. It is possible that the lesion was incorrectly designated as containing integrated DNA due to the PCR methodology used (see above). It is also possible that integration might have occurred at the 3' end of the E2 ORF with preservation of an E2 transcript detectable by mRNA ISH. As the mRNA signal from E4 was also demonstrated in this patient, this cannot be the case here and the pattern of mRNA ISH would suggest the presence of viral episomes. HPV may exist in varying states within cervical neoplasms (Matsukura *et al.*, 1989) and any coexistant episomal DNA could have resulted in the production of HPV E2 transcripts.

The 'positive-upper' pattern was demonstrated in thirteen samples from five different individuals. In these individuals all transcripts identified, including those from the high-risk E6 and E7 ORFs, were seen in the upper layers of the epithelium. Recent work on CIN suggests that E7 protein is expressed higher up the epithelium of high-grade lesions (Middleton *et al.*, 2003). However, this expression occurs as an extension of the more usual parabasal distribution of these oncoproteins. In those individuals designated as showing a 'positive upper' pattern viral transcripts for E6/E7 were not seen in the basal two-thirds of the epithelium and the pattern would not appear to represent the 'delay' in the virus life-cycle presented by Middleton and colleagues.

The focal nature of staining observed could possibly reflect variations in probe sensitivity in a particular section, but the reproducible nature of the pattern would tend to mitigate against this explanation. The fact that focal upper staining was seen across the whole panel of riboprobes for some cases raises the possibility that the RNA ISH method has threshold sensitivity and that the signal only became positive in the upper layers where the virus was present in high copy numbers following the completion of a productive life cycle. Certainly others have found transcription of both early and late viral transcripts to increase with increasing differentiation, with late viral transcripts even being identified in well differentiated regions of vulval SCCs (Park *et al.*, 1991b). The relative persistence of differentiation and cornification seen in VIN lesions could facilitate completion of the HPV life cycle at this site.

A striking observation is that all cases showing a 'positive upper' staining pattern were found to contain episomal DNA by PCR analysis. Episomal viral DNA is found at high copy numbers in the upper part of the epithelium during productive infections. It might therefore be the case that transcripts only reached readily detectable levels following transcription from large amounts of extrachromosomal viral DNA in the upper epithelium. Crum *et al.* (1989) used riboprobes from the ORFs upstream and downstream to the E1 ORF (where integration commonly occurs) to assess the origin of transcripts demonstrated in a series of biopsies from patients with CIN or VIN. The observation that the downstream probe (E2-E5-L2 region) gave at least an equivalent signal to the upstream probe (E6-E7 region) supports the notion that most of the signal observed in this study originated from transcription of viral episomes. Whilst it would appear that episomal DNA is essential for the production of late viral proteins in vegetative or productive infections (Frattini *et al.*, 1996), the contribution of episomes to malignant transformation is less clear. *In vitro* studies rather tend to reinforce the importance of integrated HR-HPV DNA in oncogenesis. The SK-v cell line was established from a lesion of VIN known to contain episomal HPV 16. In animal studies, the cell line behaves in a phenotypically similar way to the original pre-malignant lesion, but subsequent

detailed characterisation of the SK-v line has shown it to contain solely integrated DNA (Schneider-Maunoury *et al.*, 1987). Following this discovery the authors used a specific probe to show that integrants were indeed present in the clinical lesion from which the cell line was established. Furthermore, Le and colleagues (1988) were able to show that transformation of keratinocytes by a hybrid viral-human DNA fragment extracted from a HPV-positive tumour was dependent on the juxtaposition of integrated viral and cellular DNA. Nevertheless, episomal DNA may contribute to malignant progression. Recent work using organotypic raft cultures has shown that transcripts derived from episomal HPV 16 are sufficient to induce centrosome abnormalities and chromosomal instability (Duensing *et al.*, 2001b). The resulting structural damage may in turn favour viral integration and the induction of integrant derived transcripts with marked transforming capacity.

To improve the sensitivity of mRNA detection and confirm the focal transcription patterns described above, the ISH method used could be modified. Firstly, it would be attractive to have a reliable positive control with which to further optimise the RNA ISH protocol. The clinical biopsies used in these studies were limited in size and it was difficult to justify using a positive case as a control for future experiments. With the advent of organotypic raft cultures and well characterised cell lines such as the W12 line (Stanley *et al.*, 1989), it may be possible to develop a reliable and renewable source of controls for future experiments. Keratin probes and appropriate control specimens were used to guard against method failure for RNA ISH. It would perhaps have been appropriate to include a probe for a common epithelial transcript such as β-actin to control for mRNA quality within individual clinical sections. Following the use of such a control during the investigation of HPV 16 E7 transcripts in cervical SCC, van den Brule and colleagues (1991) concluded that failure to detect mRNA in the specimens was most likely secondary to mRNA degradation rather than absence of signal. Such a control might have helped in the interpretation of both the 'negative' and 'blush-speckle' patterns seen in the current series.

Using snap-frozen sections might have improved the quality of mRNA available for the *in situ*, but unfortunately such specimens are less useful for histological diagnosis and splitting the small vulval biopsies to allow freezing of one portion and formalin fixation of the other was not always practical. Labelling probes with radioisotopes and using long exposures might further enhance sensitivity. However, although these techniques have been used successfully (Crum *et al.*, 1989; Dürst *et al.*, 1992), they are time consuming, more dangerous and prone to increased background signal. More recently catalyzed reporter molecule deposition has been used to provide signal amplification without increasing background signal in ISH. The most common technique employed is that of tyramide signal amplification (TSA). Bound probe is detected using the appropriate hapten bound to horseradish peroxidase (HRP). The immobilised HRP is then used to convert labelled tyramide (the reporter molecule) into a short lived and reactive intermediate that binds to cellular proteins immediately adjacent to the bound primary antibody. A secondary antibody is then used to detect the bound, labelled tyramide. Pre-treatment of the specimens to quench endogenous peroxidase e.g. with hydrogen peroxidase, may reduce background. The technique increases the sensitivity of ISH and has even been used to detect low copy number nucleic acid sequences (Evans *et al.*, 2003).

Only three of the six SCCs studied showed a positive staining pattern following RNA ISH. In one of the cases the focal 'positive-upper' staining reported appeared to be within a focus of high-grade VIN within the invasive lesion. Two of the carcinomas showed the diffuse 'blush-speckle' pattern described above. Positive signals were only identified with both E4 and E7 riboprobes. It is therefore difficult to establish clear difference between preinvasive and invasive disease. Unfortunately, the limitations of tissue availability and time have prevented a more thorough assessment of the carcinoma specimens with the full panel of riboprobes. However, the broad spectrum of transcripts seen in the high-grade VIN lesions and in particular, the strong signals identified with E7, does not indicate a clear change in expression pattern between pre-

malignant and malignant lesions. Park et al. (1991b) demonstrated focal transcription of a full range of transcripts, including those from late genes, in well differentiated areas of 'warty' SCCs. The authors conclude that it is the degree of differentiation rather than stage of disease that determines the pattern of transcription.

A strength of the data presented in this chapter is the serial biopsies available from fourteen patients with VIN. These biopsies reveal that the pattern of viral transcription appears to remain consistent over time in lesions that demonstrated consistent histological grade. Five patients showed variations in the histological grade of lesion. It is interesting to note for two of these patients that an increased grade of VIN/VAIN was associated with a discrete rather than focal pattern of staining. Following the discussion above, it may be that the higher-grade of lesion was associated with increased viral load, which may favour such a staining pattern.

The effect of immunotherapy on viral transcription was assessed following vaccination with a viral-vector vaccine, expressing HPV 16 E6 and E7. The choice of the oncoproteins as targets for immunotherapy makes biological sense. In cervical disease, transcripts from both ORFs are commonly found in both HSILs and SCCs. The strong *in situ* signals achieved with the E7 riboprobes and the fact that E7 was identified in both pre-malignant and malignant lesions would support the use of the same targets in immunotherapy directed against vulval disease. Unfortunately, no clear effect on viral transcription was seen following successful vaccination. Of the twelve patients studied, transcription patterns observed using RNA ISH remained unchanged in ten patients over the six-month follow-up. It is however encouraging that the patient who had disease clearance following vaccination, also showed loss of mRNA signal for transcripts that had been demonstrated pre-vaccination (patient YF; samples 210/224). The loss of E7 mRNA signal in a second patient in whom partial disease regression occurred also lends support to the potential of immunotherapy as a treatment for this condition.

The data presented in this chapter have reinforced the previous suggestions of a strong association between HPV 16 infection and VIN. The virus appears to be transcriptionally active and the broad range of transcripts identified in pre-malignant lesions raises several possible targets for potential immunotherapies. The extent of transcription in VIN prevents the selection of a specific transcript pattern as a possible marker for the development of malignancy. Whether post-transcriptional modifications will affect the expression of viral proteins and thereby influence the biological effects of HPV remains to be seen. Transcriptionally active virus was identified in both normal and inflammatory biopsies and it seems likely that whilst HPV gene expression may be a driving factor early in the vulval oncogenesis, other cellular and genetic factors may be more influential at the key step of progression to invasion. Integration of HPV into the host genome was also surprisingly common in pre-malignant lesion, being demonstrated in half the HPV-positive VIN lesions tested. This finding would tend to suggest integration has a role early on in oncogenesis, and that secondary events such as genetic damage may be more influential in the progression to vulval malignancy.

Chapter 4 Genomic copy number imbalance in vulval neoplasia

4.1 Introduction

A review of the literature supports the notion of VIN as a pre-malignant condition. However, our understanding of the mechanisms behind disease progression is limited. The available effective therapeutic options are surgical and carry with them both physical and psychological morbidity. A greater understanding of the pathway from the premalignant to malignant lesion would assist in a more focussed approach to the treatment of patients, perhaps even limiting intervention to those in whom the risk of progression were high or even inevitable.

Whilst recurrent genetic alterations have been described for both CIN and cervical SCC, only limited data exist for vulval neoplasia. As has been described elsewhere (section 1.2.3.2.2.1), the majority of information available comes from small loss of heterozygosity studies using a limited panel of microsatellite markers. CGH provides an opportunity to study both gains and losses across the tumour genome. To date, only three small studies have used this technique to study vulval neoplasia (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). All these studies focussed on SCC of the vulva. By studying pre-invasive lesions (VIN), common copy number imbalances (CNI) may become apparent, supporting the notion of VIN as a malignant precursor lesion. The relative frequency of CNIs in low and high-grade VIN and vulval SCC may also be informative. Abnormalities that increase in frequency from one grade to another may reflect clonal expansion of cells with such CNIs. These abnormalities may therefore confer a selection advantage to cells that acquire them and could indicate potential markers of disease progression.

In this chapter, microdissection, DOP-PCR and CGH are used to investigate the number and frequency of CNI in 25 individuals with VIN (4 low-grade lesions and 24 high-grade lesions) as well seven individuals with SCC. The number

and site of CNI in each grade of disease will be presented in conjunction with data regarding the presence and physical state of oncogenic HPVs.

4.2 Methods

4.2.1 Microdissection and DNA extraction

As described previously (section 2.2.1), frozen 10μm tissue sections were first stained with haematoxylin and eosin before microdissection. Typically between three and five sections were microdissected, dependent upon the thickness of the abnormal epithelium. It is estimated that approximately 1000-1500 cells were retrieved for each case. All cases provided sufficient amounts of DNA for CGH as assessed by PCR for the β-globin gene (Figure 4-2). The epidermal nature of the vulval lesions studied made dissection relatively straightforward. Kallionemi *et al* have estimated that 50% contamination with non-tumour cells is the limit for detection of monosomies or trisomies (Kallioniemi *et al.*, 1994). It is very unlikely that contamination in our series exceeded 10%.

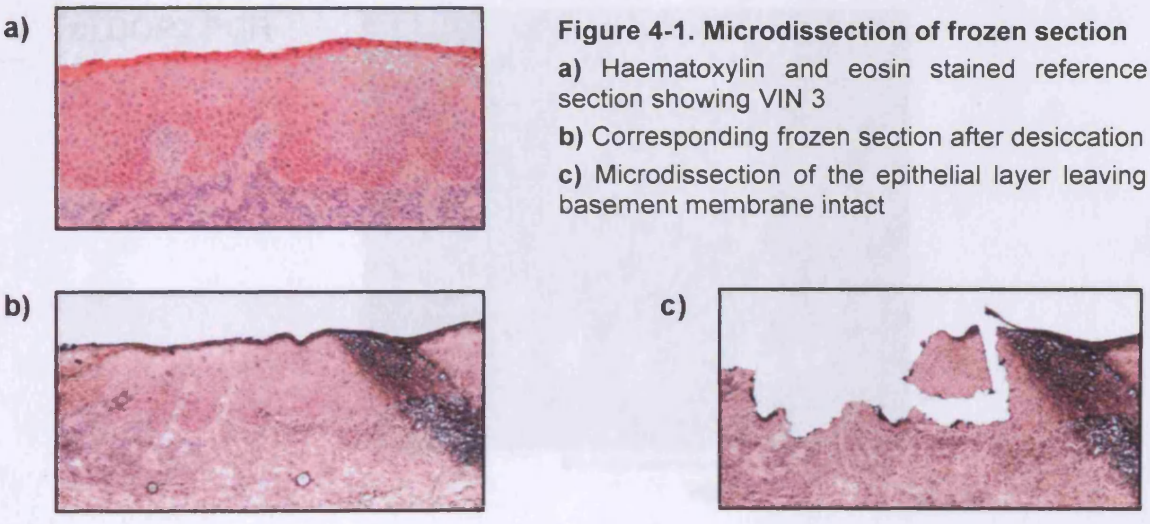


Figure 4-1. Microdissection of frozen section
a) Haematoxylin and eosin stained reference section showing VIN 3
b) Corresponding frozen section after desiccation
c) Microdissection of the epithelial layer leaving basement membrane intact

4.2.2 DOP-PCR amplification

DNA from microdissected lysates of vulval lesions was amplified and differentially labelled by DOP-PCR as described in section 2.2.4.1. Water and lysis buffer (prepared and treated in the same fashion as the DNA lysates) were used as negative controls for all primary DOP-PCR reactions. Visible product in

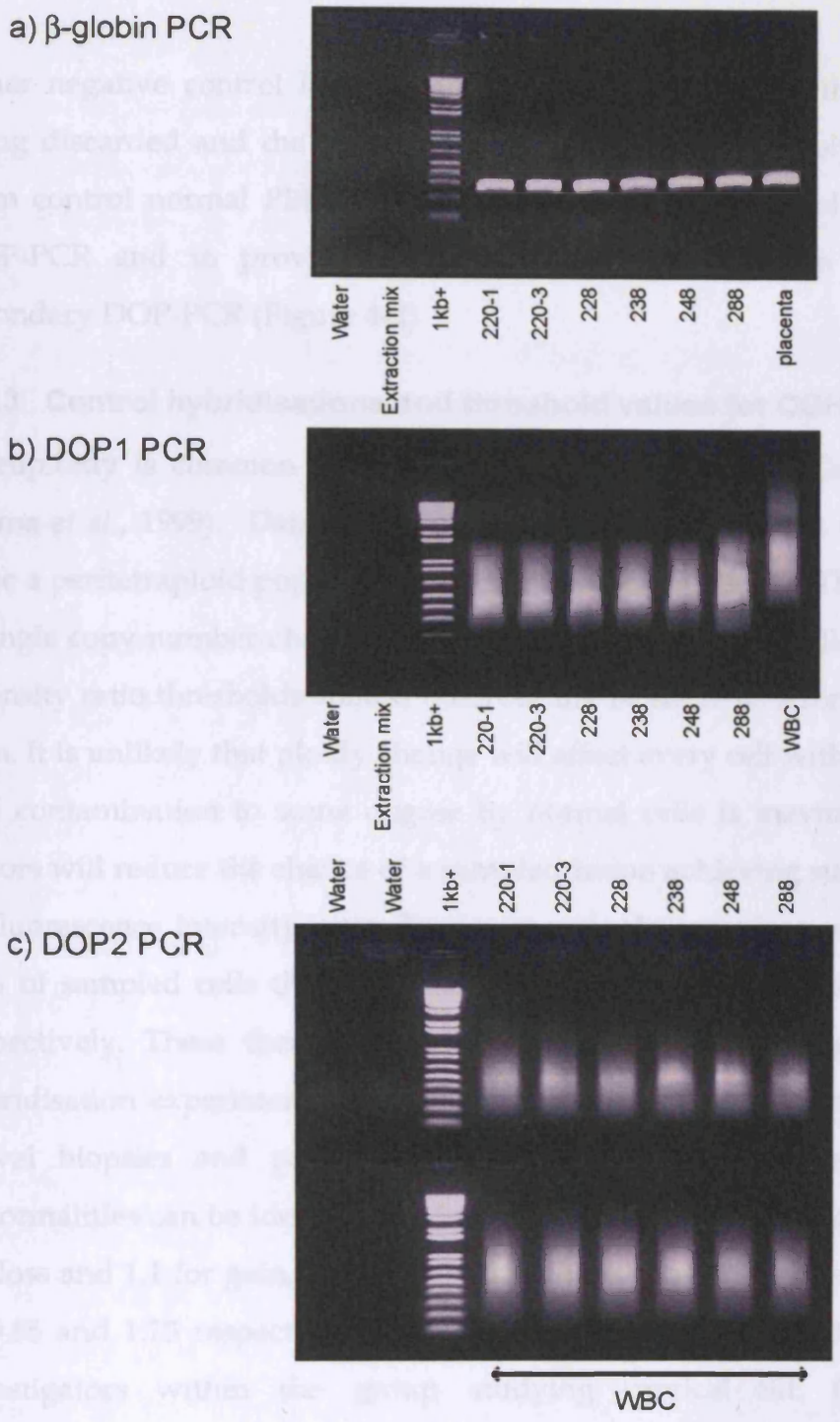


Figure 4-2. PCR products from each stage of CGH paint preparation
a) Quality of DNA obtained from microdissection assessed by β-globin PCR. Water and extraction buffer act as negative controls. Placental DNA acts as positive control.
(10µl of PCR product / 1% agarose gel)
b) Initial amplification of microdissected DNA using DOP1 PCR. Amplified DNA visible as broad smear. Water and extraction buffer act as negative controls. WBC DNA acts as positive control as well as providing template for DOP2 PCR.
(5µl of PCRproduct / 1% agarose gel)
c) Further amplification and labelling of DOP1 product using DOP2 PCR. Labelled control normal WBC DNA prepared for each test sample.
(5µl of PCR product / 1% agarose gel)

either negative control lane on gel electrophoresis led to that PCR reaction being discarded and the experiment repeated. Approximately 300ng of DNA from control normal PBMCs was used as a positive control for the primary DOP-PCR and to provide normal DNA for labelling in the subsequent secondary DOP-PCR (Figure 4-2).

4.2.3 Control hybridisations and threshold values for CGH

Aneuploidy is common in both cervical and vulval SIL (Cellier *et al.*, 1970; Lerma *et al.*, 1999). Data from cervical SIL suggest that the majority of cases have a peritetraploid population of cells (Cellier *et al.*, 1970). Therefore to detect a single copy number change present in 100% of sampled cells, the fluorescent intensity ratio thresholds should theoretically be set to 0.75 for loss and 1.25 for gain. It is unlikely that ploidy change will affect every cell within a target lesion and contamination to some degree by normal cells is inevitable. Both these factors will reduce the chance of a sampled lesion achieving such a high change in fluorescence intensity ratio. To detect a single copy loss or gain present in 50% of sampled cells these thresholds should be adjusted to 0.875 and 1.125 respectively. These theoretical limits were tested using a series of control hybridisation experiments (n=6) using tissue from both histologically normal vulval biopsies and paired control normal PBMC. Whereas false-positive abnormalities can be identified in 'normal' tissue when thresholds are set at 0.9 for loss and 1.1 for gain, no such abnormalities were seen when levels were set to 0.85 and 1.15 respectively. This finding is identical to that found by other investigators within the group studying cervical SIL (Alazawi, 2003). Thresholds of 0.85 for copy number loss and 1.15 for gain were therefore used for the subsequent analysis of lesional tissue.

4.2.4 Chromosomal regions excluded in analysis

The repeat-rich sequences found in peri-centromeric and heteterochromatic DNA can generate false positive CGH abnormalities for these regions. An attempt was made to suppress probe binding to these regions using human

placental Cot-1 DNA. These regions are highly polymorphic in copy number between individuals and suppression may therefore vary (Kallioniemi *et al.*, 1994). Ratio changes occurring within or immediately adjacent to these regions should therefore be interpreted with caution and were excluded in the analysis of this dataset.

The green and red fluorescent signals will gradually decrease towards the telomeres. As the fluorescent intensity begins to approach the level of background fluorescence, the ratio changes observed become unreliable (Kallioniemi *et al.*, 1994). This is indicated by increasing confidence intervals seen on the display karyogram (Figure 1-15). Isolated gains and losses involving the telomeres were therefore excluded from analysis.

Problems in the detection of deletions by CGH at specific chromosomal loci have also been reported, notably chromosomes 1p32-pter, 19 and 22 (Kallioniemi *et al.*, 1994). The ratio profiles at these points show significant intra-experimental variations and may occasionally be well below the average ratio, leading to the false suggestion of a deletion. In keeping with common practice, the short chromosomes 19 and 22 were therefore excluded from analysis. The reported variation in fluorescence seen towards the telomere of chromosome 1p has been attributed to this region being a gene rich area. Control DNA is usually of better quality than the test DNA and this region may therefore be better represented following DOP-PCR amplification. Once again, this may lead to the reporting of a false 'loss' for this area. However, in this series, only gains, not losses, were seen for this region. Such gains were not found in control biopsies from isolated normal vulval skin and high-level (ratio >1.5) gains were found in some individuals with high-grade disease. These facts would suggest that the copy-number gains demonstrated for 1p are valid and the data has therefore been included in the analysis.

4.2.5 Statistical methods used in analysis

Although this study includes more cases of CGH analysis in vulval neoplasia than any study published to date, the rarity of condition means that the actual

numbers involved are small. The data does not therefore meet the criteria for the application of parametric statistical tests and non-parametric methods must be employed. Fisher's exact test for the comparison of independent variables was used to compare proportions between two groups e.g. frequency of gains and losses found to affect different chromosomal arms between two pathological groups. The Mann-Whitney U test was used to compare the frequency distribution of CNIs between two pathological groups. The Kruskal-Wallis test was used to investigate overall similarities in CNIs between all the pathological groups studied. The Jonckheere-Terpstra test permitted further investigation of the order of this relationship. Data was compiled using Excel 2000® and statistical analysis was performed using SPSS 11.5®. Two-tailed tests were used and significance was assessed at the 95% level ($p < 0.05$).

4.3 Results

4.3.1 Tissue samples and patient demographics

Fresh tissue samples were obtained from patients referred to the gynaecological oncology centre and specialist vulval clinics at Addenbrooke's NHS Trust, Cambridge between April 1999 and May 2002. Prior ethical approval for this work was obtained from the local research ethics committee (LREC 98/227). The histopathological diagnosis was confirmed independently by two consultant gynaecological histopathologists. Where variation in opinion occurred (n=2), both consultants reviewed the case and reached an agreed diagnosis. Control tissue included peripheral blood mononuclear cells from normal individuals, biopsies from women with non-neoplastic vulval dermatoses and paired biopsies of adjacent normal vulval skin from women with vulval carcinoma who were undergoing wide local excision under general anaesthetic.

The median age of cases in this study was 54 years (range 37-82) and that of controls 52 years (range 33-81; p=0.960). The data for individual cohorts is shown in the Table 4-1.

Cohort	*N*	*Median age /yrs*	*Average age /yrs*	*Age range /yrs*
Control – normal	4	51	47.5	33-55
Control – assoc with neoplasia	5	72	63.8	46-81
Low-grade VIN	4	46	51.5	42-72
High-grade VIN	24	49	53.0	37-78
Vulval SCC	7	77	74.4	54-86

Table 4-1. Age distribution of participants in CGH study

The median age of patients with VIN was 49yrs and over half of the patients (15/28) were under 50yrs on recruitment to the study. Most patients (20/24) with VIN were symptomatic. The most common symptom was itch (Figure 4-3) Seventeen women had had previous treatment for their disease.

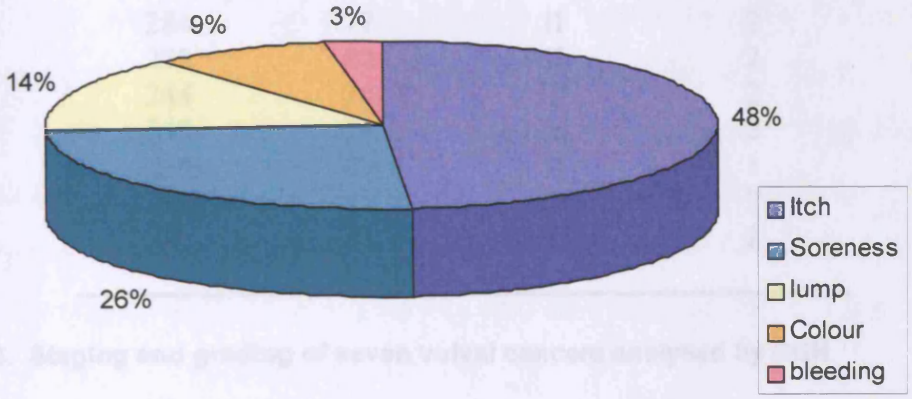


Figure 4-3. Symptoms associated with vulval intraepithelial neoplasia

The seven carcinomas included in the study were all SCCs and comprised three stage II cancers and four stage III tumours (all by virtue of lymph node metastasis). Vulval SCCs may be subdivided on the basis of the degree of differentiation of tumour cells. Grade I tumours contain no undifferentiated

cells, whilst grade II and grade III lesions contain <50% and >50% undifferentiated cells respectively.

Six of the seven tumours were moderately differentiated (grade II), whilst one of the stage II lesions was well differentiated (grade I). The staging and grade for each specimen is detailed in Table 4-2 and Table 4-3.

FIGO stage	*Description*
I	Tumour < 2cm confined to vulva
II	Tumour >2cm confined to vulva
III	Local spread to involve vagina or urethra or anus; ipsilateral lymph node metastases
IV	Local invasion into bladder or rectum; contralateral lymph node metastases; pelvic lymph nodes or distant metastases

Table 4-2. FIGO staging for SCC of the vulva

Study no.	*Age /yrs*	*Stage*	*Grade*
284	77	II	2
238	82	II	2
244	70	III	2
249	54	III	2
259B	86	II	1
274	71	III	2
281	81	III	2

Table 4-3. Staging and grading of seven vulval cancers analysed by CGH

When compared to those patients with VIN (of any grade), patients with SCC of the vulva were more likely to be over 50 years of age (7/7 vs. 12/28; p=0.009).

4.3.2 HPV status

The methodology used for HPV testing is described in detail in section 2.2.2.6. All positive PCRs were repeated at least twice. HPV type was assigned by comparison of the sequence of the PCR product with the available HPV sequences available in the HPV database of the Los Alamos National

Laboratory, University of California. Samples were assessed using the MY09/11 and GP5+/6+ primer systems, both in isolation and as a nested PCR, before being labelled as HPV negative. The majority of patients (85.7%) were infected by HPV. Data for individual cases is presented with the corresponding data for CNI in Table 4-4, Table 4-5 and Table 4-6.

The overall infection rate was significantly higher for patients with VIN than for those with carcinoma (26/28 vs. 4/7; p=0.044). The oncogenic type, HPV 16, accounted for 88.5% of infections in cases of VIN, with HPV 33 and 56 accounting for the remainder of infections. In HPV 16 positive VIN, the virus was deemed to be integrated on the basis of PCR in 65.2% of cases. Oncogenic HPV types 16 or 56 were isolated from 75% of the HPV positive carcinoma. PCR suggested HPV 16 to be integrated in 50% of HPV 16 positive vulval SCCs. Isolated infection with the LR-HPV type HPV 6b was found in one case of vulval SCC.

4.3.3 CGH analysis of vulval neoplasia

CGH analysis was performed for a total of 44 vulval biopsies (Table 4-1). The technique of CGH is described in detail elsewhere (section 2.2.4). A mean of twelve metaphases (range 9-15) was captured for each case. These were then analysed to provide a mean fluorescence intensity ratio along the length of each chromosome. CNIs detected for LSIL, HSIL and invasive SCCs are shown in the display karyograms (Figures 4-4 to 4-6) and in the summary tables (Tables 4-4 to 4-6).

It should be noted that control DNA (labelled red) was from PBMCs from a male donor with a normal karyotype by conventional banding analysis. Test DNA (labelled green) was from female subjects only. This provided an internal control for the quality of CGH hybridisation and accounts for the green signal from the X chromosome and the red signal from the Y chromosome. The CGH experiments were repeated in a proportion of the cases (8/35; 23%) confirming that the findings were reproducible.

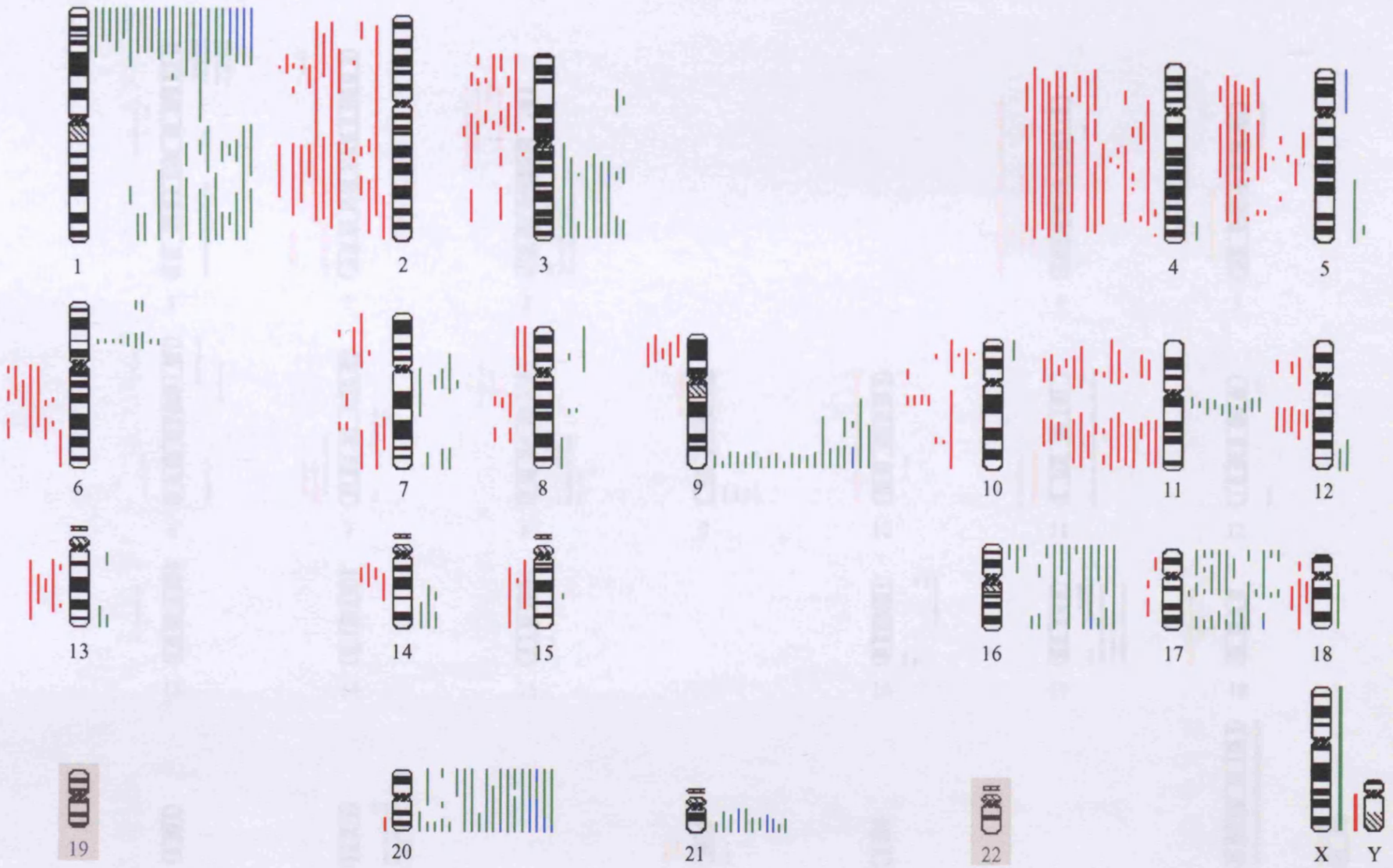


Fig 4-4. Summary Karyogram - high-grade VIN (n=24)
Copy number gains shown in green to right of chromosome with areas of high-level gain (ratio >1.5) shown in blue. Losses in red to left.

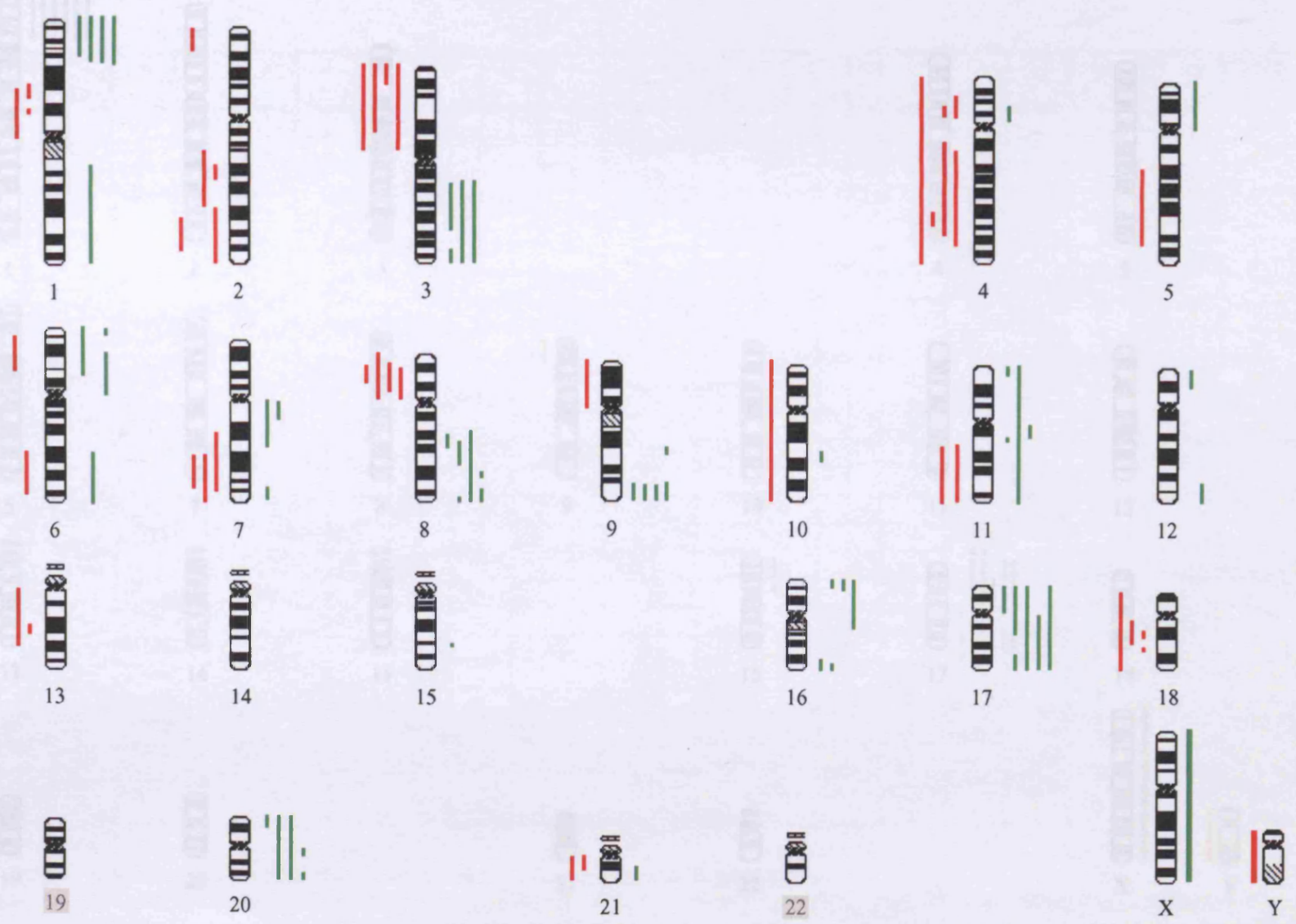


Figure 4-5. Summary karyogram - Squamous cell carcinoma of the vulva (n=7)
Copy number gains shown in green to right of chromosome. Losses in red to left.

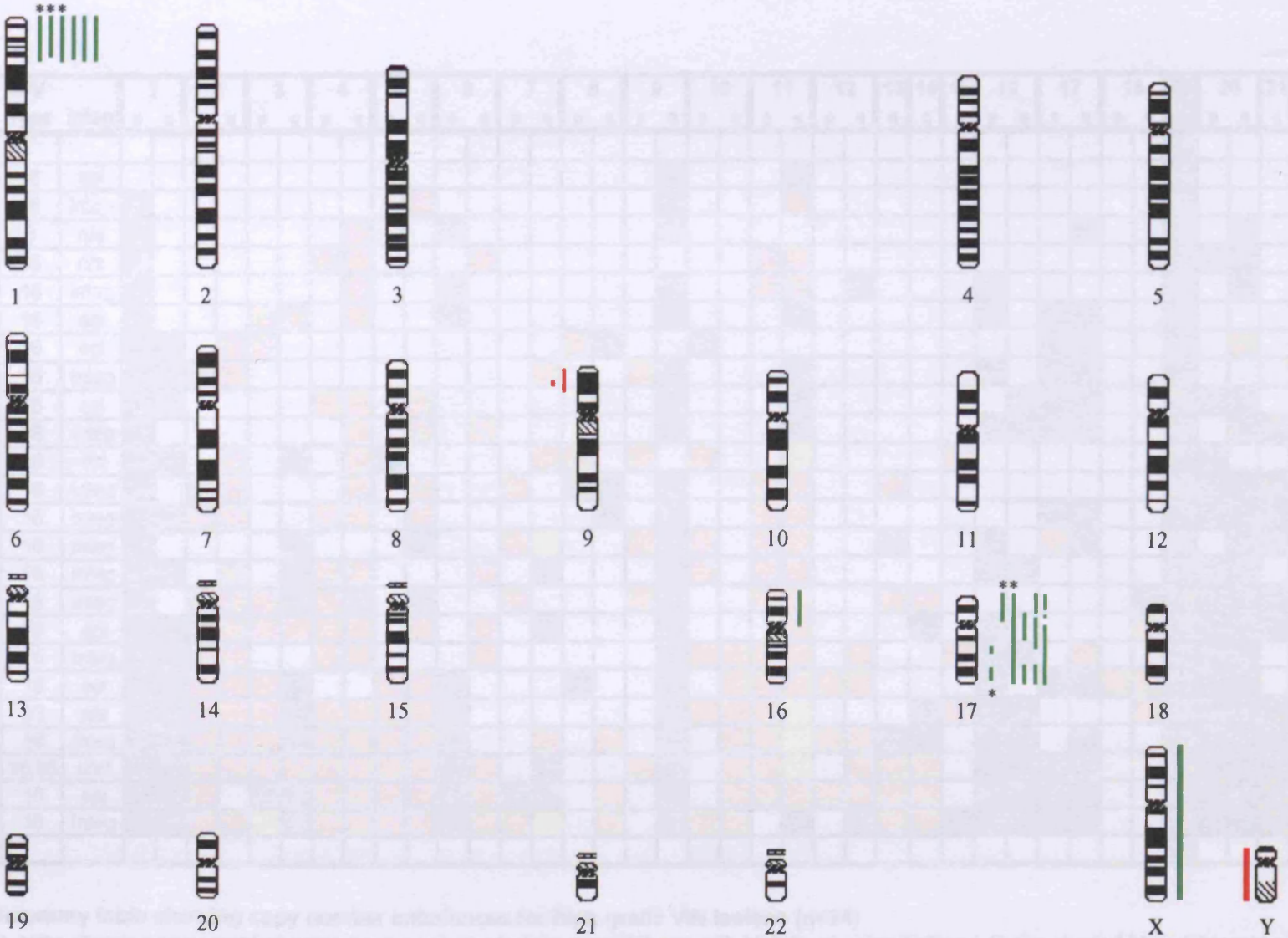


Figure 4-6. Summmary karyogram - 'Normal' associated with neoplasia (n=5, marked with *) and low-grade VIN/VAIN (n=4)
Copy number gains shown in green to right of chromosome. Losses in red to the left.

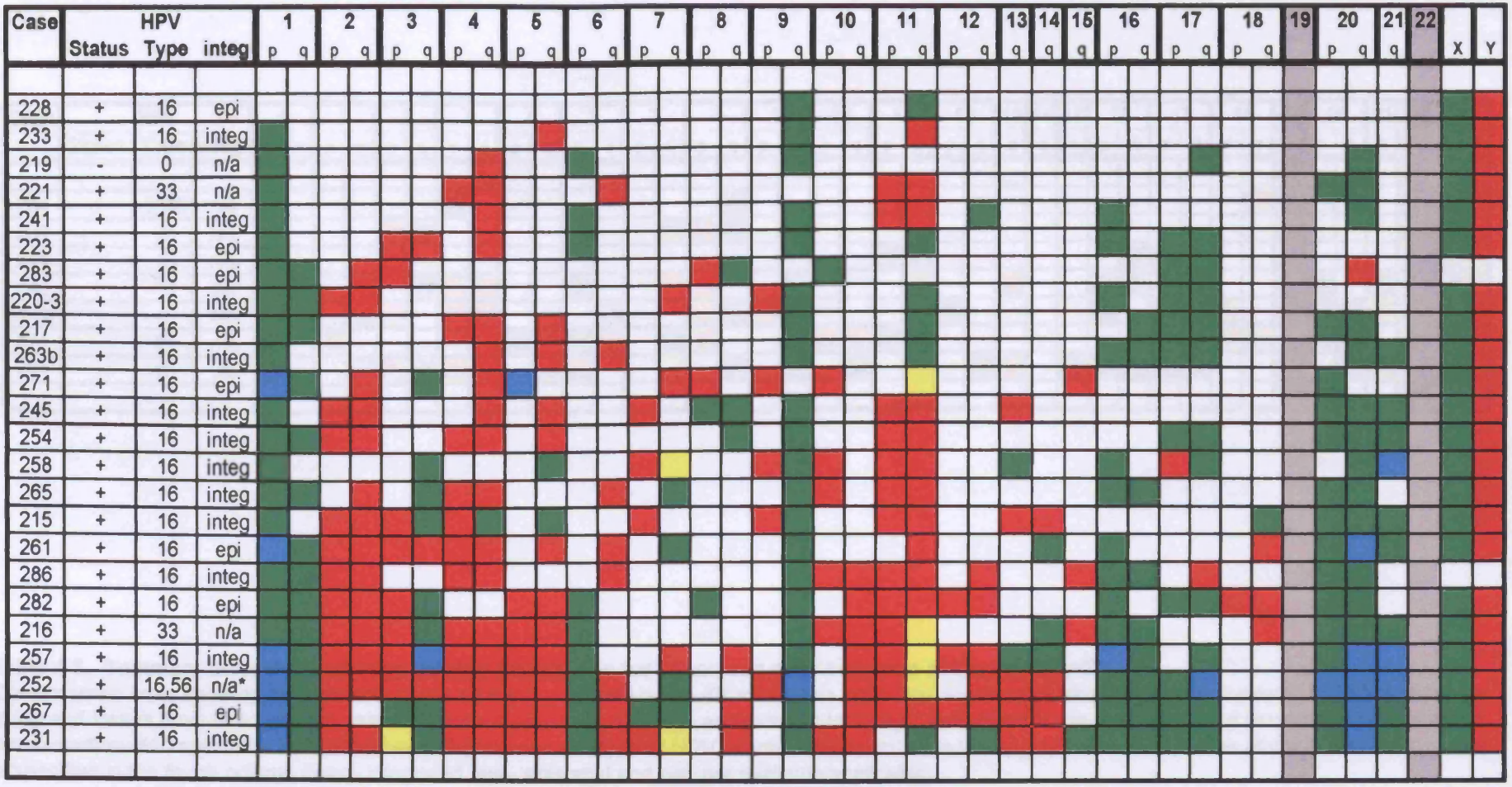


Table 4-4. Summary table showing copy number imbalances for high-grade VIN lesions (n=24)
Chromosome arms showing copy number gains have been shaded green and those with high-level gains (ratio >1.5) are shaded blue. Chromosome arms showing copy number losses are shaded red. Those with both gains and losses have been shaded yellow. Chromosomes excluded from analysis shaded grey. * indicates sample in which paired biopsy was HPV positive, but microdissected specimen HPV negative. The presence and type of HPV infection are specified in the appropriate columns. The physical state of the virus is specified in the fourth column (integ- integrated, epi - episomal and n/a- not available/applicable).

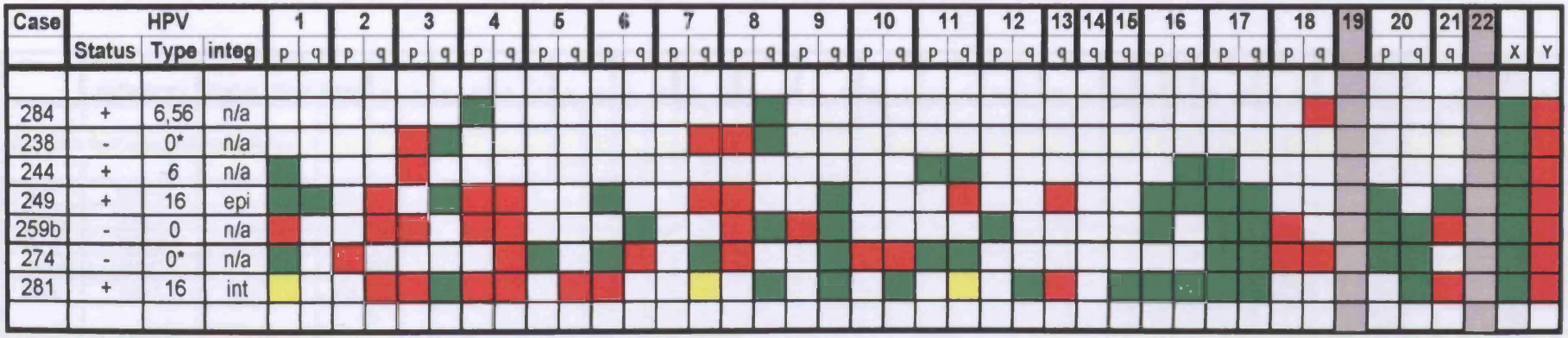


Table 4-5. Summary table showing copy number imbalances for squamous cell carcinoma of the vulva (n=7)
Chromosome arms showing copy number gains have been shaded green. Chromosome arms showing copy number losses are shaded red. Those with both gains and losses have been shaded yellow. Chromosomes excluded from analysis shaded grey. * indicates sample in which paired biopsy was HPV positive, but microdissected specimen HPV negative. The presence and type of HPV infection are specified in the appropriate columns. The physical state of the virus is specified in the fourth column (integ- integrated, epi - episomal and n/a- not available/applicable).

Case	Associated pathology	HPV Status	HPV Type	HPV Integ	1 p	1 q	2 p	2 q	3 p	3 q	4 p	4 q	5 p	5 q	6 p	6 q	7 p	7 q	8 p	8 q	9 p	9 q	10 p	10 q	11 p	11 q	12 p	12 q	13 q	14 q	15 q	16 p	16 q	17 p	17 q	18 p	18 q	19	20 p	20 q	21 q	22	X	Y
Normal' associated with neoplasia																																												
247	VIN 1	-	0	n/a																																								
263A	VIN 3	-	0	n/a																																								
254N	VIN 3	-	0	n/a																																								
281n	SCC	-	0	n/a																																								
259a	SCC	-	0	n/a																																								
Low-grade VIN/VAIN																																												
210	n/a	+	16	integ																																								
211	VIN 3	+	56	n/a																																								
220-1	VIN 3	+	16	integ																																								
247	SCC	-	0	n/a																																								

Table 4-6. Summary table showing copy number imbalances for 'normal' biopsies associated with neoplasia (n=5) and low-grade VIN/VAIN (n=4) Chromosome arms showing copy number gains have been shaded green. Chromosome arms showing copy number losses are shaded red. Those with both gains and losses have been shaded yellow. Chromosomes excluded from analysis shaded grey. Where normal epithelium was obtained from tissue adjacent to a vulval lesion, then the pathology of that lesion is indicated. The presence and type of HPV infection are specified in the appropriate columns. The physical state of the virus is specified in the fifth column (integ- integrated, epi - episomal and n/a- not available/applicable).

4.3.3.1 CGH demonstrates a consistent pattern of CNI in high-grade VIN

Twenty-four biopsies from twenty-one cases of high-grade VIN were included in the analysis. In three cases, biopsies at different time points (3-6 months apart) showed different grades of VIN and were included as distinct cases for analysis. The display karyogram (Figure 4-4) and summary table (Table 4-4) reveal the high frequency and distribution of CNI in high-grade VIN. The median number of chromosome arms involved was 15.5 per case (range 2-30). Whilst the low numbers of HPV-negative VINs prevent statistical analysis, the mean number of arms involved in CNIs was substantially higher for HPV positive lesions (16.4 arms/case; range 2-30) than was seen in the sole HPV negative case (6 arms). When HPV positive cases were considered, those with integrated HPV DNA (n=12) appeared to have marginally more CNIs per case than those in which the HPV DNA remained episomal (n=8). This difference did not reach statistical significance (median CNIs per case 16.0 (4-30) vs. 12.5 (2-29); p=0.615).

The most common gains seen were: 1p (96%); 9q (88%); 20q (75%); 20p (63%); 1q (59%); 16p (58%); 17q (54%); 21q (46%); 11q (42%); 17p (42%); 3q (38%); 6p (38%) and 16q (38%). High-level amplification (ratio >1.5) was seen in nine cases of high-grade VIN affecting 1p (six cases), 20q (five cases), 21q (three cases), 3q, 5p, 9q, 16p, 17q and 20p (one case each)

The most common losses demonstrated in high-grade VIN were: 4q (71%); 11q (67%); 2q (58%); 11p (54%); 2p (50%); 4p (50%); 5q (50%); 3p (38%) and 6q (33%).

4.3.3.2 CGH demonstrates a consistent pattern of CNI in SCC of the vulva

Seven cases of invasive SCC of the vulva were available for analysis. The median number of arms showing CNI on CGH was 18.0 (range 3-22). In the four samples demonstrated to be HPV-positive by PCR, the median number of arms showing CNI was 11.5 (range 3-22). The three HPV-negative samples showed a median of 18 arms/case (range 3-19). The most common copy number gains demonstrated were: 17p (71%); 1p (57%); 8q (57%); 9q (57%); 17q (57%); 3q (43%); 11q (43%); 16p (43%); 16q (43%); 20p (43%) and 20q (43%). The

most common losses demonstrated were 3p (57%); 4q (57%); 8p (57%); 2q (43%); 4p (43%) and 7q (43%).

4.3.3.3 Low-grade VIN/VAIN shows low frequency of CNIs as demonstrated by CGH

Four low-grade lesions were available for analysis (3 vulval and one vulvovaginal lesion). The median number of chromosomal arms affected per case was 3.0 (range 1-4). Gains were seen to affect chromosome 1p, 16p and 17. Copy number increases affecting chromosome 17 accounted for five out of the nine gains seen. Gain of 1p was also common, occurring in three of the four cases. Only two of the four cases showed losses, both affecting 9p.

4.3.3.4 The incidence of CNI varies with pathological grade of lesion

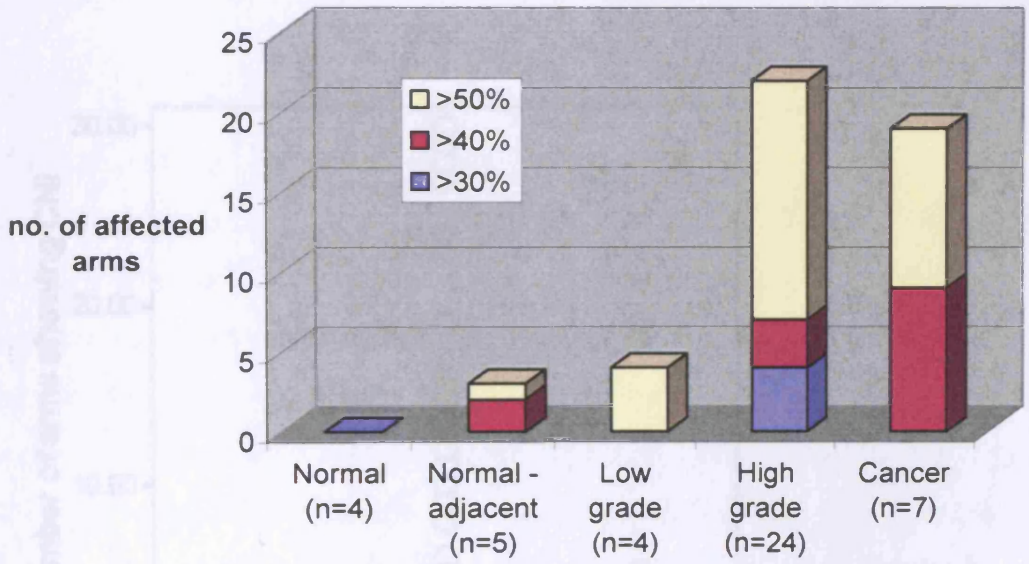


Figure 4-7. Number of chromosomal arms showing high-frequency (≥ 30%) incidence of CNI by grade of abnormality. CNIs further sub-divided into those found in ≥ 30,40 or 50% of cases.

The extent of CNI demonstrated by CGH varied according to the degree of abnormality of the vulval skin. Whilst CNI was not shown in DNA extracted from normal vulval skin biopsies, multiple chromosomal arms were affected in

cases of high-grade VIN and SCC of the vulva. CNIs were deemed to occur at high-frequency when at least 30% cases were affected. Figure 4-7 shows the number of chromosome arms affected by high-frequency CNIs in a graphical format. It can be seen that both the total number of chromosomal arms affected and the frequency with which CNI affected those arms varied between normal vulval skin, normal skin (adjacent to high-grade/SCC), low-grade, high-grade and invasive neoplasia. The overall pattern of CNI for each sub-group of vulval neoplasia is shown in Figure 4-8. Analysis of the pathological subgroups using the Kruskal-Wallis test for several independent samples demonstrated a significant difference (p=0.011) in the number of CNI per case between pathological groups (Table 4-7). There was no significant trend towards increasing number of CNIs with increasing grade of abnormality when assessed by the Jonckheere-Terpstra test (p=0.146).

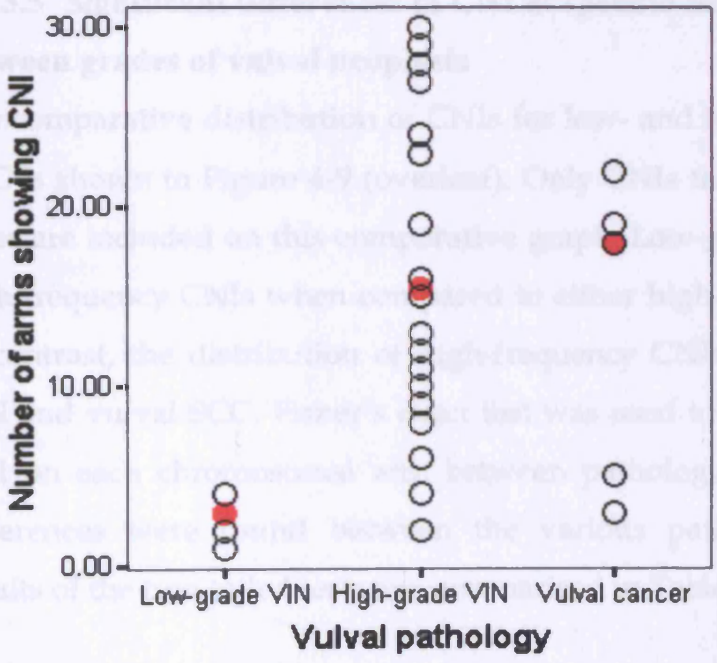


Figure 4-8. Total number of arms affected by CNI for subgroups of vulval neoplasia. Medians are shown in red.

	Low-grade VIN	High-grade VIN	Vulval cancer
N	4.00	24.00	7.00
LQ	1.25	10.00	5.00
Median	3.00	15.50	18.00
UQ	4.00	22.00	19.00
Mean	2.75	15.75	12.86
St. deviation	1.50	7.99	8.11
Kruskal-Wallis test		***p=0.011***	
Jonckheere-Terpstra test		*p=0.146*	

Table 4-7. Variation in CNIs/case between pathological subgroups. Significance difference found between groups when assessed using the Kruskal Wallis test (bold).

4.3.3.5 Significant differences in CNI at specific chromosomal loci were seen between grades of vulval neoplasia

The comparative distribution of CNIs for low- and high-grade VIN and vulval SCC is shown in Figure 4-9 (overleaf). Only CNIs that were found in ≥ 30% of cases are included on this comparative graph. Low-grade VIN shows far fewer high frequency CNIs when compared to either high-grade VIN or vulval SCC. In contrast, the distribution of high-frequency CNIs is similar for high-grade VIN and vulval SCC. Fisher's exact test was used to compare the frequency of CNI on each chromosomal arm between pathological subgroups. Significant differences were found between the various pathological subgroups. The results of the two-tailed tests are summarised in Table 4-8.

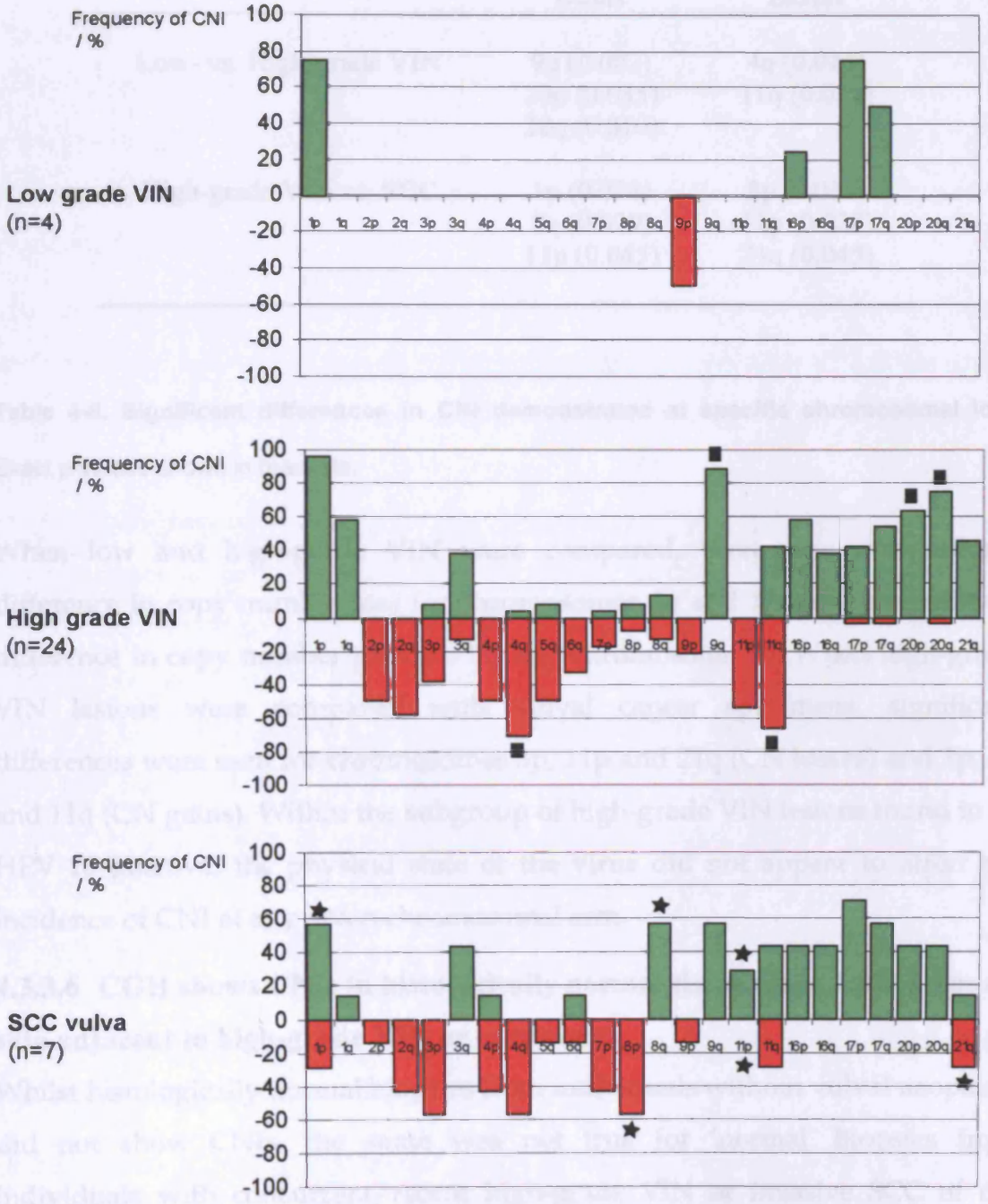


Figure 4-9. Comparative high frequency copy number imbalances for low grade VIN, high grade VIN and SCC of the vulva

Charts show the percentage of cases exhibiting CNI (gains shown as positive in ■, losses shown as negative in ■). Selected chromosomal arms shown are those where frequency of CNI exceeds 30% in at least one sample group. Arms showing significant differences between low- and high-grade VIN are indicated by ■ . Arms showing significant differences between high-grade VIN and SCC are indicated by ★ .

Pathological groups compared	*Significant difference in CNI*	
	Gains	*Losses*
Low- vs. High-grade VIN	9q (0.002) 20p (0.035) 20q (0.010)	4q (0.016) 11q (0.024)
High-grade VIN vs. SCC	1p (0.028) 8q (0.029) 11p (0.045)	8p (0.014) 11p (0.025) 21q (0.045)

Table 4-8. Significant differences in CNI demonstrated at specific chromosomal loci. Exact p-values shown in brackets.

When low and high-grade VIN were compared, there was a significant difference in copy number loss for chromosomes 4q and 11q and a significant difference in copy number gain for 9q and chromosome 20. When high-grade VIN lesions were compared with vulval cancer specimens, significant differences were seen for chromosomes 8p, 11p and 21q (CN losses) and 1p, 8q and 11q (CN gains). Within the subgroup of high-grade VIN lesions found to be HPV 16 positive, the physical state of the virus did not appear to affect the incidence of CNI at any given chromosomal arm.

4.3.3.6 CGH shows CNIs in histologically normal tissue taken from areas of skin adjacent to high-grade VIN or carcinoma

Whilst histologically normal biopsies from individuals without vulval neoplasia did not show CNIs, the same was not true for 'normal' biopsies from individuals with concurrent/recent high-grade VIN or invasive SCC of the vulva (Figure 4-6). Of the five such cases analysed, CNIs were demonstrated in three. These were all gains affecting chromosome 1p or chromosome 17 (17p and 17q equally affected).

4.4 Discussion

Data on the genetic changes associated with vulval neoplasia are limited. To date only three studies have used CGH to establish recurrent patterns of genetic abnormalities in a small number (n=36 total) of SCCs of the vulva (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). No studies of CGH in VIN have yet been published. This study includes 28 cases of VIN and 7 cases of vulval SCC and may therefore provide a valuable insight into the genetic abnormalities associated with vulval neoplasia.

The cohort of women studied appears to be representative of patients suffering with VIN and vulval carcinoma. Over half of the women with VIN were under 50yrs of age, which lends some support to the arguments of other authors (Herod *et al.*, 1996; Jones *et al.*, 1997; Joura *et al.*, 2000; Thuis *et al.*, 2000) who feel that the incidence of VIN may be increasing in younger women. Given this age distribution and the basaloid/warty histological nature of the lesions studied, it was not surprising to find that over 90% of cases of VIN were positive for HPV. The predominance of HPV16 infection is in keeping with previous data. HPV infection was not however confined to younger patients. Three of the twenty-six cases of VIN positive for HPV infection were over 70 years, which is in agreement with the findings of Haefner and colleagues (1995) who found HPV-positive VIN occurred into the eigth decade. The majority of patients with VIN were symptomatic, although VIN occurred in asymptomatic women in 16.7% of cases. This finding emphasises the potential importance of inspection of the vulva, particularly within a high-risk group such as those women attending the colposcopy clinic for abnormal cervical cytology.

Seven cases of vulval carcinoma were available for analysis. Patients with carcinoma were more likely to be over 50 years of age than patients with high-grade VIN (11/24 vs. 7/7; p=0.025). The identification of an isolated LR-HPV infection (type 6b) is unusual but has been documented previously (Kasher *et al.*, 1988). It should be noted that the association of such infections does not indicate a causal role for this virus type in the development of carcinoma.

It was extremely difficult to obtain normal vulval epithelium for control hybridisations. Previous LOH studies of cervical intraepithelial neoplasia have used cervical stroma as control normal tissue, but the different anatomy of the vulva does not easily provide for such a control. The sensory innervation of the vulva necessitates the administration of local anaesthetic prior to taking a biopsy. This fact, coupled with the psychosexual morbidity associated with vulval surgery, means that biopsies are not taken as readily as may be the case in cervical disease. For these reasons 'normal' biopsies were usually obtained either as opportune specimens where the clinical suspicion of pathology was incorrect and the subsequent histology proved normal; or from benign inflammatory dermatoses where confirmatory histology was being sought; or as a paired specimen where the subject was undergoing an excisional treatment under anaesthetic for an adjacent pathology e.g. SCC of the vulva. Although histologically unremarkable such epithelium may not be otherwise normal. There is evidence from the study of X chromosome inactivation (Tate *et al.*, 1997; Rosenthal *et al.*, 2002) and microsatellite markers (Rosenthal *et al.*, 2002) that VIN is usually monoclonal and that vulval SCC may share the same monoclonal origins as adjacent VIN (Rosenthal *et al.*, 2002). Furthermore, such clonal expansion may occur before the development of cytological atypia (Tate *et al.*, 1997; Lin *et al.*, 1998). It is therefore perhaps unsurprising that three of the four 'normal' biopsies taken from patients with concurrent high-grade lesions or invasive disease showed CNI. The abnormalities detected were all gains and affected chromosome 1 and/or chromosome 17. The finding of aneusomy affecting chromosome 17 is in keeping with data from an earlier study, which used chromosome-specific FISH to identify chromosome 17 aneusomy in vulval neoplasia (Carlson *et al.*, 2000). Not only was such aneusomy common in vulval carcinoma, but it could also be identified in biopsies from histologically normal or inflamed tissue adjacent to such carcinomas. These findings support the concept of aneuploidy as a driving force at an early stage of vulval carcinogenesis.

In order to overcome the relative paucity of true normal control biopsies it would have been possible to use archival material from the pathology tissue bank. However, a strength of this study is the quality of DNA available from fresh frozen biopsies. Variations in fixation time and processing techniques involved in producing paraffin embedded sections may result in DNA damage which has been shown to adversely affect the yield achieved with DOP-PCR amplification (Speicher *et al.*, 1993). The optimum length of DNA probe of 600-2000bp is more readily achieved with the large amounts of high quality DNA that can be extracted from frozen tissue sections. To ensure consistent high quality CGH experiments a decision was therefore made to limit this study to frozen tissue specimens. It is both probable and plausible that lack of CNIs demonstrated in the true normal biopsies studies is likely to be reflective of normal vulval skin. Furthermore, work on other sites in the lower genital tract would support this. Using the same methodology, Alazawi (2003) failed to demonstrate CNIs in nine control normal cervical biopsies.

CNIs were a common finding in both high-grade VIN and vulval carcinoma. Three previous studies using CGH in vulval carcinoma have examined 10, 18 and 8 cases of vulval SCC respectively (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). All found fewer CNIs per case than were demonstrated in this study - mean CNIs/case 2.6 (0-11), 3.2 (0-5) and 5.3 (2-17) respectively vs. 12.9 (3-22). Whilst the higher fluorescence intensity ratio thresholds used by Allen *et al.* (> 1.2 for gain and < 0.80 for loss) and Micci *et al.* (> 1.25 for gain and < 0.75 for loss) may explain the lower frequency of CNIs seen in these studies, the previous work by Jee *et al.* used similar fluorescence intensity ratio thresholds to those chosen in this thesis (>1.17 for gains and <0.85 for losses). Contamination by normal cells could reduce the sensitivity of CGH to detect CNIs of either low frequency or amplitude. It is difficult to assess the precise methods of microdissection used in these earlier studies. It would appear that Jee and colleagues scraped epithelium from unstained 10μm sections using a scalpel blade (Jee *et al.*, 2001). In the work presented in this thesis, frozen sections were first stained with haematoxylin and eosin before microdissection

with a 23G needle. Great care was taken to avoid contamination with any normal cells from around the lesion of interest and this could have improved the sensitivity to detect CNIs. Such precision has been associated with higher than usual CNIs per case in a study of cervical SILs (Aubele *et al.*, 1998). It should be noted that control normal hybridisations (n=6) did not show any evidence of CNIs and that several of the common gains were found as high level gains in some cases, supporting the CNIs seen as true results. Furthermore, the four cases of low-grade VIN studied showed far fewer CNIs per case (median 3.0, mean 2.75 (range 1-4)) suggesting that there is not a simple threshold effect and that CNIs vary with the degree of vulval neoplasia. A further possible explanation for the high frequency of CNI found in VIN is the possibility of selection bias affecting the VIN case mix for this study. All patients were recruited at a tertiary referral centre through specialist vulval and gynaecological oncology clinics. Although patients can also be referred direct from local primary care providers, many have been treated for some time at other units and are sent to the centre for help with resistant disease. As such, the cases studied might well reflect established VIN, which may well be genetically unstable and have had time to develop multiple abnormalities.

The frequency of CNIs per case was significantly different between the pathological subgroups (Kruskal-Wallis test; p=0.011). There was no significant trend of increasing CNIs with increasing grade of neoplasia (Jonckheere-Terpstra test; p=0.146). The two pathologists reviewing the biopsies showed excellent agreement in their grading of intra-epithelial lesions, but this is not always the case. Preti and colleagues used a panel of sixty-six vulval biopsies to show significant interobserver variation in the use of the current three-tiered grading system between six consultant pathologists (Preti *et al.*, 2000). The authors found that when VIN 2 and 3 were combined into a single 'high-grade' class interobserver agreement in diagnosis was more likely. Other authors have suggested that in practice a two-tiered grading system is already in operation and should therefore be considered for routine clinical use (van Beurden *et al.*, 1999). Such a classification would be in keeping with the Bethesda system

already in place for CIN (Solomon *et al.*, 2002). The data presented here suggest that it may also be biologically appropriate to consider such a two-tiered grading system for VIN. High-grade VIN lesions show significantly more CNIs per case than the low-grade lesions (median CNIs/case 15.5 (2-30) vs. median 3.0 (1-4); p=0.003). In contrast there was no significant difference between the VIN 2 and VIN 3 subgroups of the high-grade cohort (median CNIs/case 19.0 (9-30) vs. 15.0 (2-29); p=0.521). Therefore, where CNIs are concerned, a two-tiered grading system for VIN would seem to be acceptable.

CNIs in low-grade lesions were limited to gains affecting chromosome 1p, 16p chromosome 17 and loss affecting 9p. All of these abnormalities were seen in high-grade lesions. The relative low numbers of low-grade cases makes it difficult to draw firm conclusions regarding the relevance of these abnormalities with regard to disease progression. The high frequency of gains seen on chromosome 1p and 17p in both high and low-grade lesions, as well as in normal skin adjacent to VIN 3 or carcinoma, suggests that these abnormalities may occur early on in the neoplastic process. Gain in 1p was seen in almost all cases of high-grade VIN. As has been stated previously, VIN appears to be clonal. It is therefore likely that gain in 1p is acquired from the low-grade precursor lesion and may be important in the prediction of progression risk in low-grade disease.

High-grade VIN shows high numbers of both gains and losses affecting a wide variety of chromosomal arms. All but one case was positive for HR-HPV infection. When HPV16 positive cases are considered, there was no significant difference in the frequency of CNI between those in which the virus was episomal and those where it was integrated (n=8; median CNI/case 12.5 (2-29) vs. n=12; median CNI/case 16.0 (4-30); p=0.615). As has previously been described (section 1.2.1.5), loss of E2 expression as a result of integration may result in loss of transcriptional control of the high-risk oncoproteins E6 and E7 (Schwarz *et al.*, 1985; Cripe *et al.*, 1987), a reduction in apoptosis (Webster *et al.*, 2000) and immune modulation (Alazawi *et al.*, 2002). One might therefore expect integration to be associated with greater genetic instability and increased

numbers of CNIs. Whilst PCR for the E2 gene was found to correlate accurately with the physical state of the virus as assessed by Southern blotting (Das *et al.*, 1992), the technique will fail to identify cases in which the virus is present in both integrated and episomal forms. The inclusion of cases as episomal in which the virus is predominantly integrated might therefore mask any real difference between the two groups. The use of alternative methods to assess viral integration, such as the amplification of papillomavirus transcripts (APOT) technique, might help to identify such mixed populations (Klaes *et al.*, 1999). Integration usually results in disruption or deletion of the viral early-region polyadenylation signal. Transcripts derived from integrated E6/E7 oncoproteins will therefore show viral sequences at their 5'-ends and cellular sequences at their 3'-ends. In contrast, the most common E6-E7 viral transcript from episomal DNA is spliced at the E1-donor site signal to the E4-splice acceptor site and is terminated at the viral polyadenylation site (Schwarz *et al.*, 1985; Sherman *et al.*, 1992). APOT uses RT-PCR to distinguish between integrated and episomal viral transcripts on the basis of these structural differences at the 3'-ends. Amplification by nested PCR allows the distinction between integrant-derived and episome-derived transcripts on the basis of a size difference. Accepting the limitation of target stability inherent to any RNA-based technique, APOT could provide further functional information, in that only samples containing transcriptionally active HPV will test positive.

The common CN gains seen in this series of patients with high-grade VIN have are described in detail elsewhere (section 4.3.3.1. and Figure 4-4). Whilst no published CGH data exist for patients with VIN, several of these gains (namely 1p, 1q, 3q, 6p, 9q, 11q, 16q, 17p, 17q and 20q) were found in one of more of the earlier series of CGH performed on vulval cancers (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). High-level gains (ratio >1.5) were seen for at least one case in the majority of the regions commonly seen in this study, suggesting that they were most likely to be true gains. Observed gains affecting 20p have not previously been demonstrated in vulval neoplasia but have been shown in CGH studies of CIN and cervical SCC (Heselmeyer *et al.*, 1996; Heselmeyer *et*

al., 1997b; Dellas *et al.*, 1999; Kirchhoff *et al.*, 1999; Matthews *et al.*, 2000; Kirchhoff *et al.*, 2001; Rao *et al.*, 2004).

Gain affecting 1p has already been discussed in the context of low-grade disease and histologically normal tissue adjacent to VIN 3/SCC. This aberration (consensus region 1p32 -pter) was seen in almost every high-grade lesion and over half the carcinomas (p=0.028). In addition, a quarter of high-grade VINs showed high-level gain affecting this region. In contrast, neither Allen *et al.* nor Jee *et al.* found any gains on 1p in twenty-eight vulval carcinomas. However, in a recent small series of eight SCCs investigated with CGH, similar aberrations were found in three cases (Micci *et al.*, 2003). This lower reported incidence of gain of 1p affecting SCCs is in keeping with the current series. The selection advantage conferred by gain in 1p may be important for the transition from low- to high-grade VIN. It is more difficult to explain the reduced frequency of gain in 1p in SCCs compared to high-grade VIN, although it is possible that genetically unstable lesions could lose this CNI as they progress to carcinoma.

Aneusomy of chromosome 17 in high-grade VIN and SCC, as well as normal and inflammatory tissue adjacent to such lesions, has been demonstrated using chromosome specific FISH (Carlson *et al.*, 2000; section 1.2.3.1). It is interesting that whilst most samples were found to show gains in CN, loss of chromosome 17 was found in 5% of samples (n=110) and was significantly associated with HPV-positive VIN 3. Almost all the VIN 3 patients in the current study were shown to be HPV-positive using PCR. Despite this, CN gains affecting chromosome 17 (p and/or q) were far more common than losses. The proposed association cannot therefore be confirmed. Several studies have focussed on LOH around the p53 locus (chromosome 17p13.1) and such loss has been linked to the development of HPV-negative vulval SCCs (Flowers *et al.*, 1999; Rosenthal *et al.*, 2001). As has been described previously, a major function of the viral oncoproteins from HR-HPV is to target cellular p53 (section 1.2.1.4), so there would be little selection pressure to develop a mutation affecting this TSG in the presence of HR-HPV infection. Gain affecting chromosome 17 has been found in CGH studies of cervical cancer specimens (Heselmeyer *et al.*, 1996;

Dellas *et al.*, 1999), *in vitro* studies of HPV-transfected cells (Solinas-Toldo *et al.*, 1997) and cervical cancer cell lines (Solinas-Toldo *et al.*, 1997; Harris *et al.*, 2003) and SCCs at other anatomical sites (Voravud *et al.*, 1993). Amplifications affecting both arms of chromosome 17 are also common to some other neoplasms including osteosarcoma (Lau *et al.*, 2004) and breast cancer (Wu *et al.*, 2000). Many potential oncogenes are located on chromosome 17, including ERBB2 (17q11.2-12) which was found to be amplified in seven of fifty cervical carcinomas studied (Mitra *et al.*, 1994).

Gain of 3q has been reported in both published CGH studies of vulval SCC (Jee *et al.*, 2001; Allen *et al.*, 2002) as well as a recent cytogenetic study of vulval and vaginal tumours (Micci *et al.*, 2003). In our study the gain of 3q often involved the whole arm, both for cases of VIN and vulval SCC. The smallest area of gain appeared to be 3q21-23, which is in keeping with Allen and colleagues' finding in HPV-positive SCCs of the vulva (Allen *et al.*, 2002). In five of the nine VIN cases showing gain of 3q and two of the three cases of vulval SCC, this gain was accompanied by loss on 3p. The loss on 3p tended towards loss of the whole arm.

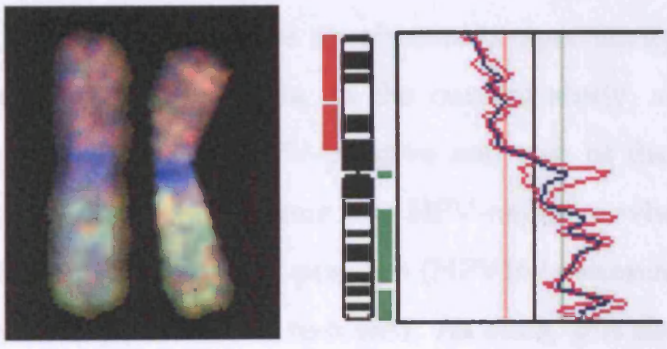


Figure 4-10. Possible isochromosome formation involving chromosome three. Copy number loss affecting short arm indicated by red fluorescent signal. Copy number gain affecting long arm indicated by predominance of green fluorescence. Chromosome pair from case 281.

Together, these changes suggest the possibility of isochromosome formation with loss of 3p and retention of 3q (Figure 4-10 above). The previous studies of

SCC of the vulva found these two abnormalities to be common (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). Other authors have described similar changes in CIN and SCC of the cervix (Heselmeyer *et al.*, 1996; Dellas *et al.*, 1999; Kirchhoff *et al.*, 1999; Matthews *et al.*, 2000; Kirchhoff *et al.*, 2001; Rao *et al.*, 2004). The relative frequencies of gain in 3q between CIN and early invasive SCC of the cervix led Heselmeyer and colleagues to propose that the gain of 3q was an early event that defined the transition from pre-invasive to invasive disease (Heselmeyer *et al.*, 1996). However, the finding of the same gain in 2/9 cases of CIN 2 suggests that its presence does not indicate inevitable progression to carcinoma (Kirchhoff *et al.*, 2001). Nevertheless, the recurrent nature of gain in 3q and its presence at higher frequency in invasive carcinoma than in pre-invasive disease (both in vulval and cervical disease) would support an important role for this region in carcinogenesis of the lower genital tract. It is worth noting Allen and colleagues found the gain of 3q to be exclusive to HPV positive tumours (Allen *et al.*, 2002). Furthermore, the cervical tumours assessed by Heselmeyer *et al.* were HPV-positive (Heselmeyer *et al.*, 1996) and 3q gain was common to three of four HPV-positive cell lines assessed *in vitro* (Solinas-Toldo *et al.*, 1997). This gain is also seen in HPV-positive anal cancer and its precursor lesions (Heselmeyer *et al.*, 1997a; Haga *et al.*, 2001) leading to the suggestion that gain of 3q could be an aberration specifically associated with HPV-related anogenital oncogenesis. In the current study, all the high-grade VINs exhibiting this gain were HPV-positive and two of the three SCCs also harboured the virus. The third tumour was HPV-negative when assessed using the microdissected DNA, but HPV positive (HPV16/episomal) when a paired biopsy from the same patient was re-tested. As such, this data would further support the role of 3q gain in HPV-related neoplasia of the lower genital tract.

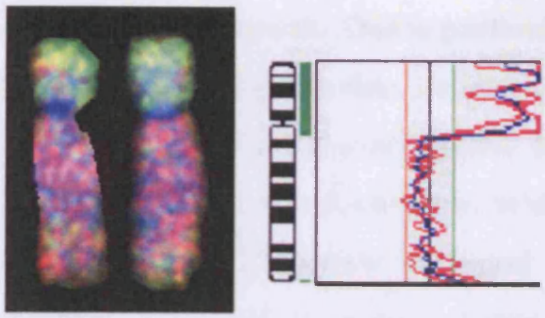


Figure 4-11. High-level gain of 5p. Short arms of chromosome 5 clearly show predominantly green fluorescence compatible with copy number gain in the tumour. Threshold for gain set at 1.15. Fluorescence ratio exceeds 1.5 indicating high-level copy number gain. Sample chromosome pair taken from case 271.

Gain in 5p was seen in one case of high-grade VIN (high-level gain, ratio >1.5) and one case of invasive carcinoma. This gain has previously been demonstrated in invasive vulval carcinoma (Allen *et al.*, 2002; Micci *et al.*, 2003), but not in pre-invasive disease. In cervical neoplasia, gain in 5p is commonly found in advanced cervical carcinoma, often present as a high-level gain or amplification (Heselmeyer *et al.*, 1997b; Kirchhoff *et al.*, 1999; Rao *et al.*, 2004). *In vitro* work confirms this aberration is common in both HPV-transfected immortal keratinocytes (Solinas-Toldo *et al.*, 1997) and cervical cancer cell lines (Solinas-Toldo *et al.*, 1997; Dowen *et al.*, 2003; Harris *et al.*, 2003). Studying the HR-HPV containing W12 cell line has demonstrated isochromosome 5p formation at medium to late passage (Dowen *et al.*, 2003), as the cell line acquires an invasive phenotype (Pett *et al.*, 2004). In the face of this evidence, the finding of 5p gain in high-grade VIN must however be interpreted with caution. The source biopsy for this lesion (study no. 271) was taken during a wide local excision for a known poorly differentiated carcinoma, occurring on a background of high-grade VIN. Histological assessment of the fresh frozen biopsy failed to demonstrate any invasion, even when reassessed by an unblinded pathologist in the light of the CGH result. Frozen sections are inferior in quality to paraffin-embedded sections with regard to tissue

morphology and histological assessment. This is particularly true for those that have been desiccated prior to microdissection. As invasive foci are a relatively common finding in VIN 3 (section 1.1.3) it is possible that the adjacent frozen sections used for CGH contained foci of invasion, which were missed at the time of microdissection and inadvertently included. However, it remains possible that this aberration occurred in an area of true preinvasive disease. In this case one could conclude that acquisition of gain in 5p might occur earlier on in vulval carcinogenesis than is the case in SCC of the cervix. The lesion from case 271 does appear highly genetically unstable with thirteen arms affected by CNI, two by high-level gains and may be an extreme case.

Several novel high frequency gains were noted in this series of high-grade VINs, specifically gains on 20p, 21q and 11q. Gains affecting 20p, including high-level gains, have been demonstrated in SCC of the cervix (Heselmeyer *et al.*, 1997b; Rao *et al.*, 2004) and in CIN 2 (Kirchhoff *et al.*, 2001). In addition, Allen and colleagues found gain in 20p in one of eighteen SCCs of the vulva (Allen *et al.*, 2002). The gains seen in VIN and vulval carcinoma in this series were far more common and were frequently associated with gain of 20q. This seems most likely to reflect aneusomy of this chromosome. This region has been found to be similarly affected in squamous cell carcinomas at other sites, including oral, oesophageal and gastric cancers (Struski *et al.*, 2002) and in other tumours of the genital tract (Sonoda *et al.*, 1997). *In vitro* studies within our group using the W12 HR-HPV cell line have shown that gain of 20p is a common event and is associated with immortalisation of the cells[4].

Gain in 21q has not been previously described for either vulval or cervical neoplasia. Indeed, for SCCs affecting the head and neck this region more usually exhibits loss (Struski *et al.*, 2002) and may be associated with poor prognosis (Yamamoto *et al.*, 2003). Two of the seven vulval carcinomas assessed in this thesis showed a similar loss for this region and Allen *et al.* documented loss in one of their eight HPV-negative vulval carcinomas (Allen *et al.*, 2002).

[4] Dr. Mark Pett, MRC Cancer Cell Unit, Hutchinson MRC, Cambridge, personal communication, 2004

However, high-level gains were evident in three of the eleven cases of high-grade VINs, supporting the validity of CN gain in this region. The relative infrequency of this aberration in SCC of the vulva (1/7 cases) suggests that the CNI may occur as a consequence of genetic instability associated with high-grade VIN. Genetic loss from this region may be more important than gain in the development of invasive carcinoma.

CGH studies of genital neoplasia have previously shown losses affecting chromosome 11 (p and q) in vulval carcinoma (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003) and in both pre-invasive and invasive squamous neoplasms of the cervix (Herod *et al.*, 1996; Heselmeyer *et al.*, 1996; Kirchhoff *et al.*, 1999; Matthews *et al.*, 2000; Harris *et al.*, 2003; Rao *et al.*, 2004). CGH data is supported by LOH studies in vulval carcinoma (Flowers *et al.*, 1999; Pinto *et al.*, 1999; Rosenthal *et al.*, 2001; Rosenthal *et al.*, 2002) and cervical cancer (for review see Lazo, 1999). These studies suggest the potential of TSGs both on the short and long arms of chromosome 11. This concept is further supported by animal work with SiHa and HeLa cell lines, where transfer of normal chromosome 11 to these cells prevents their usual tumorigenic action in nude mice (Saxon *et al.*, 1986; Koi *et al.*, 1989). Loss affecting 11p and 11q was common in high grade VIN. Although ratio profiles for 11p approached fluorescence intensity ratio thresholds for the SCCs in this thesis, loss was only seen affecting 11q in the carcinomas.

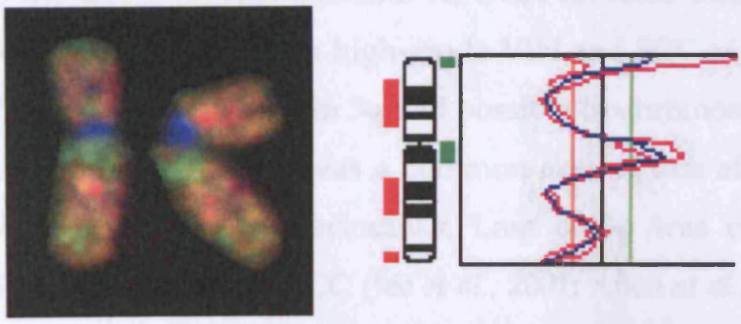


Figure 4-12. Pattern of copy number loss and gain affecting chromosome 11. Red fluorescence clearly visible affecting short and long arms. Small subcentromeric area of gain seen as distinct area of green fluorescence. Sample chromosome pair from case 252.

A striking feature of both high-grade VIN was the frequent finding of a small area of gain on chromosome 11q (11q12.1-13.5), between the two areas of loss on 11p and 11q. This pattern was often obvious at the time of inspection of the metaphase down the fluorescence microscope (Figure 4-12). This area also showed gain in two of the seven vulval carcinomas studied (a third case showed gain of the whole chromosome). Gain on 11q has previously been demonstrated in molecular cytogenetic studies of HPV-transfected (Solinas-Toldo *et al.*, 1997) and cervical cancer cell lines (Solinas-Toldo *et al.*, 1997; Harris *et al.*, 2003). Allen *et al.* found gain in a similar region of 11q by CGH in two HPV-negative vulval SCCs (Allen *et al.*, 2002), but this has not otherwise been reported in other CGH studies of lower genital tract neoplasia. The 11q13 locus contains several potential oncogenes including CCND1, which encodes cyclin D1 and EMS1, which encodes a filamentous actin binding protein. Copy number gains have been mapped to this locus for breast and oesophageal carcinoma, where such gains have been associated with poor prognosis (Hui *et al.*, 1997; Yen *et al.*, 2001). Whilst there are no data to support these genes as oncogenes in vulval neoplasia, it should be noted that classical cytogenetic studies have shown breakpoints occurring in this region (Worsham *et al.*, 1991; Micci *et al.*, 2003) and *in vitro* studies of cervical cancer cell lines have shown high level amplifications affecting 11q13 (Harris *et al.*, 2003).

In addition to the losses on chromosome 11, CGH revealed other non-random pattern of CN losses affecting both high-grade VIN and SCC of the vulva. The association of loss of 3p with gain in 3q and possible isochromosome formation has been alluded to earlier. This was a common area of loss affecting 38% of high-grade VINs and 57% of carcinomas. Loss of 3p was common to all previous CGH studies of vulval SCC (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003) and is also in agreement with microsatellite-based LOH studies of both VIN and vulval carcinoma (Flowers *et al.*, 1999; Pinto *et al.*, 1999; Rosenthal *et al.*, 2001). Allen *et al.* found loss on 3p to be more frequent in HPV positive SCCs (Allen *et al.*, 2002), whilst two of the LOH studies found the reverse to be

true (Flowers *et al.*, 1999; Rosenthal *et al.*, 2001). In the current study, loss of 3p was common in HPV-positive high-grade VIN and found in both HPV-positive and negative carcinomas.

Loss on chromosome 4 (p and q) was a common finding in both high-grade VINs and SCCs. Previous CGH studies show a conflicting pattern for this loss. Although loss on 4p was the most common aberration found in the small study by Jee *et al.*, the other two studies have found this change in far fewer carcinomas (Allen *et al.*, 2002; Micci *et al.*, 2003). Evidence from *in vitro* studies suggests that chromosome 4 contains genes important in promoting cellular senescence that are lost in squamous cell carcinoma (Ning *et al.*, 1991; Loughran *et al.*, 1997; Forsyth *et al.*, 2002). One possible mechanism for this senescence may be repression of telomerase (Backsch *et al.*, 2001).

Copy number loss on chromosome 2 was common in the current series. Loss affecting 2q has been documented by several authors in both CIN and invasive cervical SCC (Heselmeyer *et al.*, 1996; Heselmeyer *et al.*, 1997b; Kirchhoff *et al.*, 2001; Rao *et al.*, 2004). Loss in the current series was a mixture of discrete loss affecting 2p and/or 2q and loss of the entire chromosome suggestive of aneuploidy. The frequency of copy number loss was much higher than has previously been reported (Allen *et al.*, 2002; Micci *et al.*, 2003). Classical cytogenetic studies of vulval SCC have implicated chromosome 2q as a common region for structural chromosomal rearrangements (Worsham *et al.*, 1991; Teixeira *et al.*, 1999; Micci *et al.*, 2003). An *in vitro* study has suggested that the introduction of chromosome 2 to SiHa cervical cancer cells induces senescence (Uejima *et al.*, 1995). Loss of chromosome 2 may therefore be another mechanism of promoting immortality. More recent work by the same group has implicated a region on 2q and, in contrast to chromosome 4, the induction of senescence appears to be independent of telomerase (Uejima *et al.*, 1998).

Many of the abnormalities identified in the seven cases of invasive carcinoma were also found in high-grade VIN. There was a significant difference in gain of 8q between SCC of the vulva and high-grade VIN ($p=0.029$). This was a

common gain in all of the previous series of CGH in vulval cancer (Jee *et al.*, 2001; Allen *et al.*, 2002; Micci *et al.*, 2003). In contrast to Allen and colleagues who found this gain to be more common in HPV-negative cancers, gain of 8q was spread evenly between HPV-positive and negative tumours. This region shows amplification in a variety of tumours including both SCCs and other cell types (Struski *et al.*, 2002) and has been shown to contain the proto-oncogene MYC (8q24) which is commonly amplified in SCC of the cervix (Ocadiz *et al.*, 1987). Allen *et al.* suggested the smallest area of gain was centromeric to this proto-oncogene (Allen *et al.*, 2002). In the current study, gains often affected most of the arm and were associated with loss of 8p suggesting the possible formation of an isochromosome (Figure 4-13). Micci et *al.* found a high frequency of such an isochromosome in their assessment of 35 SCCs of the vulva using classical cytogenetic techniques (Micci *et al.*, 2003).

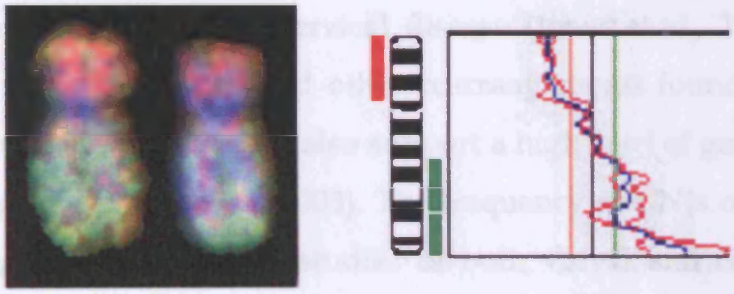


Figure 4-13. Chromosome 8 showing possible isochromosome formation. Copy number loss affecting short arm of chromosome 8 visible as red fluorescent signal whilst gain affecting long arm indicated by green fluorescence. Sample chromosome pair from case 259B.

The low numbers of SCCs assessed make it difficult to compare subdivisions within the group. The frequency of CNI (arms/case) suggests the possibility of two subgroups (Figure 4-8), with four cases showing high numbers of CNIs (8-22 arms/case) and three cases relatively few CNIs (3-5 arms/case) This difference may simply reflect the low sample number, but it is worth noting that three of four patients in the high CNI group had metastatic disease (Stage III) compared to one of three patients in the low CNI group. It is not possible to

assess CNI according to stage of the vulval SCCs previously reported, as the authors do not provide this information.

VIN lesions demonstrate frequent CNIs, many of which are shared by invasive carcinomas. This finding provides further support for high-grade VIN as a truly malignant precursor lesion. The regions showing CNIs in VIN and SCC of the vulva are similar to those of their cervical counterparts. Ideally, selective abnormalities demonstrated in the current work could be confirmed using interphase cytogenetics (reviewed by Wolfe *et al.*, 1997). The frequency and incidence of high-level gains is much greater in high-grade VIN than CIN/cervical SCC (Heselmeyer *et al.*, 1996; Kirchhoff *et al.*, 1999), suggesting that vulval neoplasia may in fact be more genetically unstable than cervical neoplasia. This finding would fit with the clinical observation that the time of progression from high-grade disease to invasive carcinoma may be much shorter in untreated VIN than cervical disease (Jones *et al.*, 1994). The high frequency of isochromosomes and other rearrangements found by Micci and colleagues in vulval cancer would also support a high level of genetic instability in vulval neoplasia (Micci *et al.*, 2003). The frequency of CNIs observed in this study was higher than in other studies of both vulval and cervical disease. However, the results were reproducible, control hybridisations were normal and high-level gains were found for many of the observed regions supporting the validity of my findings. Low-grade lesions appear to show far fewer CNIs than high-grade VIN. The findings in high-grade lesions give little reassurance to clinicians faced with the problem of managing women with such lesions. Many of these lesions appear to be highly unstable showing multiple CNIs with high-level gains and amplifications. In some cases changes that have been associated with invasive lesions and/or poor prognosis at other sites (e.g. loss of 3p/gain of 3q, gain in 5p, gain in 11q) are evident in histologically pre-invasive lesions. Coupled with the clinical reality of occult invasion in high-grade disease (Chafe *et al.*, 1988; Hørding *et al.*, 1995; Modesitt *et al.*, 1998; Husseinzadeh *et al.*, 1999; Thuis *et al.*, 2000), non-surgical management of high-grade VIN should therefore be approached with caution. However, whilst this

work has helped to increase our understanding of the genetic changes associated with vulval carcinogenesis, it remains to be seen if the aberrations identified have functional consequences. The availability of data from the human genome project and high-throughput array techniques should allow further investigation based on the novel findings presented here.

Chapter 5 Therapeutic vaccination as a treatment for high-grade vulval and vaginal intraepithelial neoplasia

5.1 Introduction

The high prevalence of HPV infection in high-grade VIN has led investigators to postulate that immunotherapy targeting virally infected cells might be of use in treating this debilitating condition. The immune response to HPV has been considered in detail elsewhere (section 1.2.2). For established disease, the induction of a cell-mediated cytotoxic response appears necessary to produce disease regression by clearance of virally infected cells. Such a response is dependent on both the presentation of viral antigens to immune effector cells and the development of a local cytokine milieu that favours a cytotoxic response. The cytokine modulator Imiquimod is effective in the treatment of benign viral warts and has already been used to treat small numbers of patients with high-grade VIN (section 1.1.7.3.3). Inducing clinically effective HPV-specific CMI remains a highly desirable goal. In this chapter, the safety, immunogenicity and efficacy of a recombinant vaccinia candidate vaccine will be described.

5.1.1 TA-HPV - a recombinant vaccinia vaccine

This phase II clinical trial described in this chapter used the TA-HPV recombinant vaccinia vaccine from Xenova Group plc, Cambridge[5]. TA-HPV is derived from the Wyeth strain of vaccinia, which was widely used in the smallpox vaccination campaign. The virus has an established safety profile, is relatively easy to manipulate and is effective at generating a cell-mediated immune response. The construction and characterisation of TA-HPV has been described elsewhere (Boursnell *et al.*, 1996). The vaccine contains the fused E6 and E7 ORFs from HPV 16 and 18, each under the control of a vaccinia promoter (Figure 5-1). These two HPV types were chosen as they rank amongst the most common in HPV-associated lower genital tract neoplasia. The E6 and

[5] formerly Cantab Pharmaceuticals plc.

E7 oncoproteins appear to be central to HPV related oncogenesis (section 1.2.1.4) and the ORFs are known to be transcribed in both high-grade cervical (Shirasawa *et al.*, 1991) and vulval intraepithelial lesions (Park *et al.*, 1991b; van Beurden *et al.*, 1995). The E7 gene of both HPV types was first modified by mutation of the retinoblastoma gene product binding sequence so that there can be no binding of the resultant viral protein with retinoblastoma protein. The fused E6 and modified E7 genes have previously been demonstrated to show no transforming activity. The lytic, rather than persistent, nature of the vaccina infection further adds to the safety of the construct. A persistent local infection might promote tumour development at the vaccination site. A region in the HPV 18 E6 coding sequence was further modified to remove a potential signal for vaccinia virus early transcription (Borysiewicz *et al.*, 1996; Boursnell *et al.*, 1996; Kaufmann *et al.*, 2002). The virus was prepared at a concentration of 1 x 10^8 plaque-forming units/ml and stored securely at -80°C

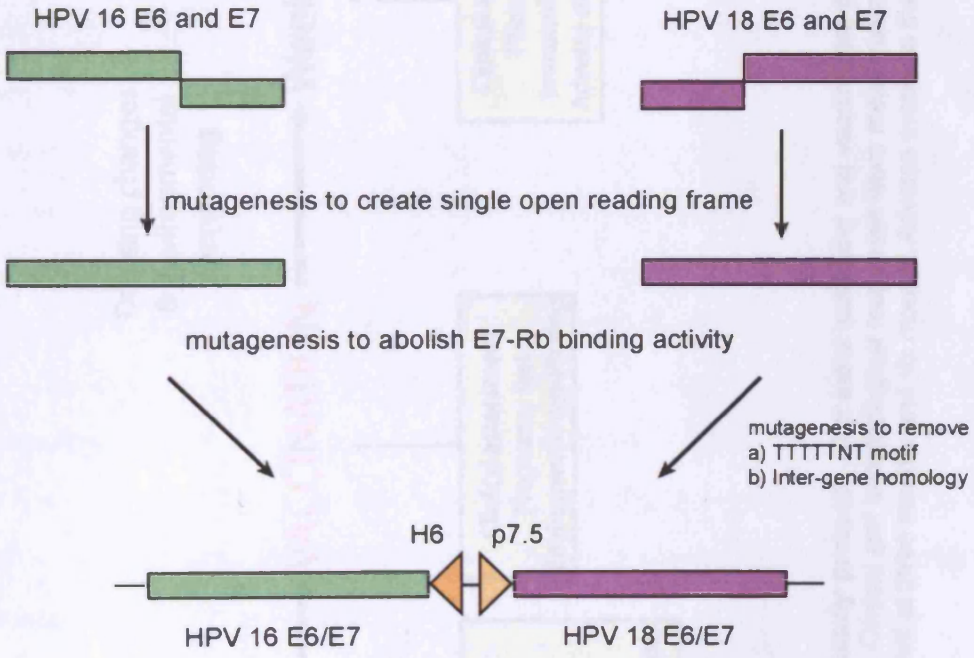


Figure 5-1. Construction of the recombinant vaccinia vaccine TA-HPV. High-risk ORFs from HPV 16 and 18 fused and modified before insertion into Wyeth vaccinia strain under the control of vaccinia promoters.

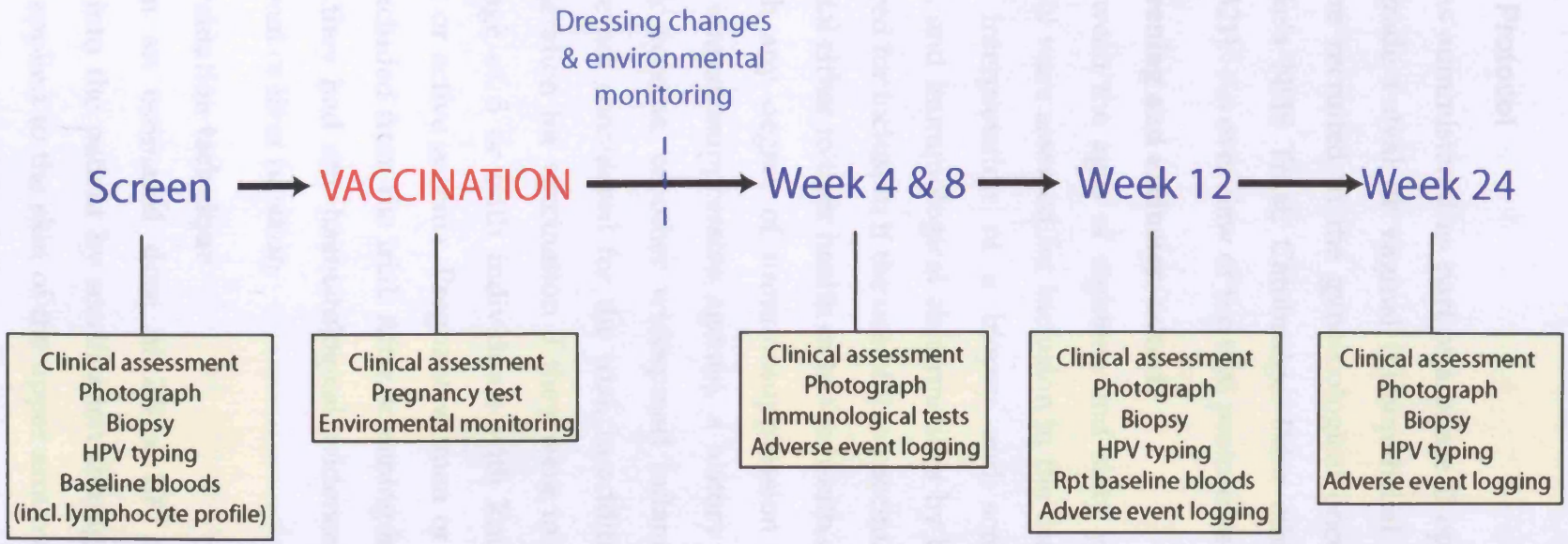


Figure 5-2. TA-HPV study protocol. After initial screening and vaccination, patients were followed monthly for three months and then for final review at six months. Clinical and immunological responses were measured at each visit and a repeat biopsies were taken for HPV typing and histological assessment at three months and six months. Adverse events to the vaccination were sought at each of the trial visits.

5.2 Methods

5.2.1 Trial Protocol

TA-HPV was administered as part of a phase II open labelled trial in subjects with high-grade vulval or vaginal intraepithelial neoplasia (VIN or VAIN). Patients were recruited in the gynaecological oncology and vulval clinics at Addenbrooke's NHS Trust, Cambridge. Prior ethics approval was obtained (LREC 99 /321). An overview of the trial protocol is given in Figure 5-2.

5.2.1.1 Screening and exclusion criteria

Women between the ages of eighteen and sixty years with high-grade VIN, VAIN or AIN were assessed for inclusion in the trial. They were diagnosed by histological interpretation of a biopsy and screened for haematological, biochemical, and immunological abnormalities by blood testing. Women were not considered for inclusion if the use of live vaccinia virus could be expected to be detrimental either to their health or to the health of their close contacts. Thus, women with any degree of immunosuppression (including those receiving therapeutic immunosuppressive agents), a history of severe allergic reaction, active atopic eczema, or other widespread inflammatory or exfoliative skin disease were not considered for the trial. In addition, women were excluded from consideration for vaccination if they were in close contact with children under the age of 5 or with individuals with known or suspected immune suppression or active eczema. Pregnant women or those at risk of pregnancy were also excluded from the trial. After screening investigations, patients were excluded if they had any haematological evidence of immunosuppression or abnormal renal or liver function.

5.2.1.2 Vaccination technique

TA-HPV (in an estimated dose of 2.5 x 10^5 plaque-forming units) was introduced into the patient by scarification through a 50µl drop of the virus suspension applied to the skin of the upper arm overlying the deltoid muscle. The area was allowed to dry before covering with a waterproof dressing (Opsite

Plus, Smith & Nephew). The dressing was changed twice a week until the scab, which formed as a result of the virus-induced inflammation, had separated and the vaccination site was healed. Samples for virological detection were taken from the surface of the dressing every week before its removal to test for the risk of possible contamination of the environment, and on separation, the final scab was tested for the presence of live virus. As a genetically modified vaccine, the use of TA-HPV was subject to prior approval by the Gene Therapy Adivisory Committee (GTAC) of the Department of Health, the National Health and Safety Executive (HSE) and the Medicines Control Agency (MCA).

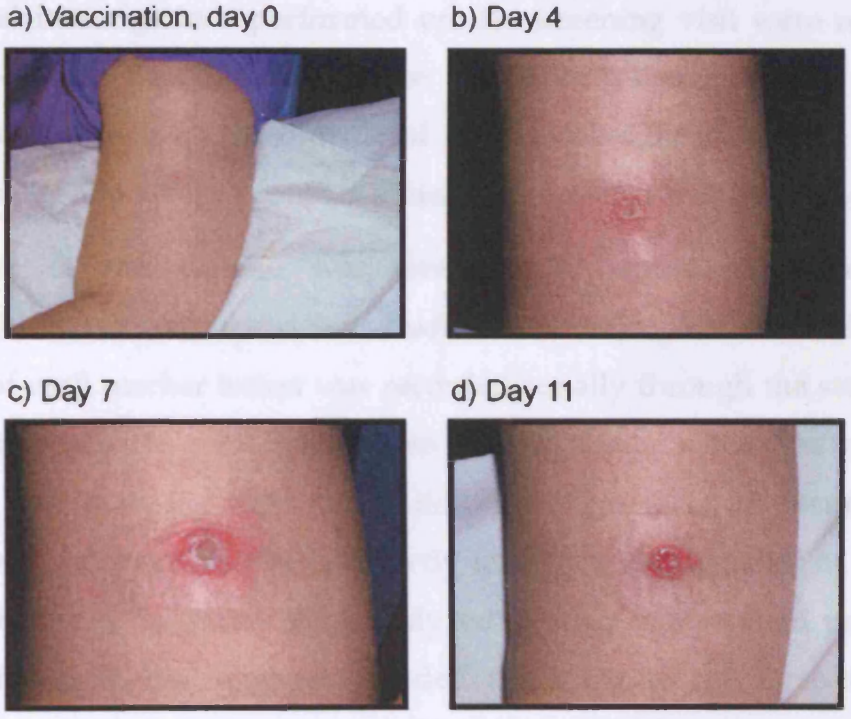


Figure 5-3. The local response/take to a successful vaccination with TA-HPV. a) Initial vaccination by scarification through a 50μl drop of TA-HPV applied to the skin over the deltoid region of the arm. **b)** Typical blister formation, associated with viral replication, usually occurs within 2-4 days. Subject may develop mild temperature and axillary lymphadenopathy. **c)** Blistering/erythema commonly maximal at around day 7. **d)** As viral replication decreases, scab forms at blister site. When left uncovered, scab typically separates between day 10 and 21 leaving a small scar at the vaccination site. Due to virus containment with an occlusive dressing, the time to scab separation was longer (median 26.5 days, range 15-32 days).

5.2.2 Follow-up

After vaccination, all subjects were followed for a six-month period. Trial visits were initially monthly until three months, followed by a final review at six months. No other treatment for the genital intraepithelial neoplasia was given during the trial or in the month preceding the vaccination. Data was collected to allow the assessment of vaccine safety as well as clinical, virological and immunological responses over the course of the study period.

The occurrence of adverse events was documented at each visit during the trial period. In addition to the clinical assessment, the haematological and biochemical investigations performed on the screening visit were repeated at the three-month visit to investigate possible systemic effects following vaccination. Swabs from the outside of the occlusive dressing were tested for the presence of live virus to confirm virus containment during the study.

The extent of the disease was assessed by direct measurement and photographic record of up to two marker lesions in each case. The longest diameter of each marker lesion was recorded serially through the study. Three biopsies of the visible areas were taken at time points: before the vaccination and at 12 and 24 weeks after vaccination. Care was taken to ensure that the biopsies were taken so as not to directly influence the diameter of the lesion being studied. The biopsies were analyzed by two independent pathologists and the intraepithelial neoplasia graded according to the three-tier ISSVD classification. The presence of HPV detectable by polymerase chain amplification in tissue was sought at each biopsy point. Any symptomatic change was recorded with particular reference to irritation and pain. Assessment was by subjective grading of symptoms into one of four categories: nil; mild; moderate; or severe. After vaccination, subjects were actively questioned to ascertain the nature of any systemic symptoms potentially attributable to vaccination, and all additional medications were recorded. PBMCs and serum samples were taken prior to and following vaccination on a monthly basis for the first three months. These samples were used for immunological testing using ELISPOT to assess the development of

HPVspecific CTL responses and ELISA to assess the antibody response to the vaccinia vector (section 2.2.5).

5.2.2.1 Collaborators

Patients were recruited, vaccinated and followed up by the author under the clinical supervision of Dr. J. Sterling, Consultant Dermatologist, Addenbrooke's NHS Trust. HPV typing was performed by the author. Pathological assessment of the tissue biopsies was performed by Dr. R. Moseley and Dr N. Coleman, Department of Histopathology, Addenbrooke's NHS Trust. The TA-HPV vaccine construct was supplied by Xenova Group Plc., Cambridge. HLA typing was performed on serum samples by the Xenova Group Plc and the data analaysed by the Immunology group headed by Professor P. Stern of the Paterson Institute of Cancer Research at the Christie Hospital, Manchester. Vaccine and HPV specific immune responses were measured in collaboration with two groups: Mr. C. Boswell, Xenova Group plc, Cambridge, UK and Dr. S. van der Burg, Tumour Immunology Group, University of Leiden, The Netherlands. The author and Dr J. Sterling were responsible for collating and interpreting data from this study.

5.3 Results

5.3.1 Patients, HPV and HLA typing

Twelve women were recruited for vaccination (Table 5-1). The age range was 42-54 years with a median of 47.5 years. The diagnosis of AGIN had been made between 3 months and 14 years (mean, 3.4 years) before the start of the vaccine trial. The disease was multifocal in eight women. All women had high-grade anogenital intraepithelial neoplasia. Eleven women had VIN (grade III) and one woman (no. 5) had VAIN (grade II). Previous treatments received by half the patients included excisional surgery, laser ablation, and topical imiquimod cream. All patients were positive for HR-HPV, with eleven of the twelve women infected by HPV-16. HPV-33 was identified in one woman (no. 10). Ten of the patients were HLA-A2 haplotype.

Study number	*Age*	*Diagnosis*	*Duration of disease*	*HPV type*	*HLA-A type*
1	48	VIN 3	11 years	16	2,68
2	49	VIN 3	8 years	16	2,3
3	47	VIN 3	1 year	16	2,24
4	49	VIN 3	2.5 years	16	2,3
5	44	VAIN 2	3 months	16	2,3
6	47	VIN 3	6 months	16	1,2
7	42	VIN 3	1 year	16	2,32
8	49	VIN 3	2 years	16	1,24
9	45	VIN 3	3 months	16	2,26
10	42	VIN 3	4 months	33	2,24
11	52	VIN 3	14 years	16	2
12	53	VIN 3	3 months	16	3

Table 5-1. Patients enrolled in TA-HPV vaccination study

5.3.2 Vaccine safety

Vaccine safety was assessed in terms of both the risk to the individual patient and the potential risk to the environment of virus escape.

5.3.2.1 Adverse events

There was no detectable adverse effect on kidney, liver, or bone marrow function as assessed by blood samples and in no patient was there a significant increase in disease area or progression. All patients remained generally well during and after the vaccination. A local reaction at the site of vaccination at approximately 7-10 days was common but temporarily limited arm movement in only two patients. A small area of scarring was left at the vaccination site.

5.3.2.2 Virus containment

The swabs taken from the outside of the dressing each week before its removal showed no evidence in any case of escape of the active virus from beneath the dressing to the exterior. A total of fifty-seven swabs from twelve patients were assayed, and no virus particles were detected in any of the samples. Occlusive dressings were worn over the vaccination site until the scab formed, dried and then separated from the skin. Gauze samples from under each dressing were

tested for the presence of live TA-HPV. Live virus was detected in the under surface of the dressing in all patients after vaccination for a mean of 21 days. In five of the twelve patients, live virus was detected in the final sample containing the scab. In seven patients, no live virus was detected in the final sample.

5.3.3 Clinical response

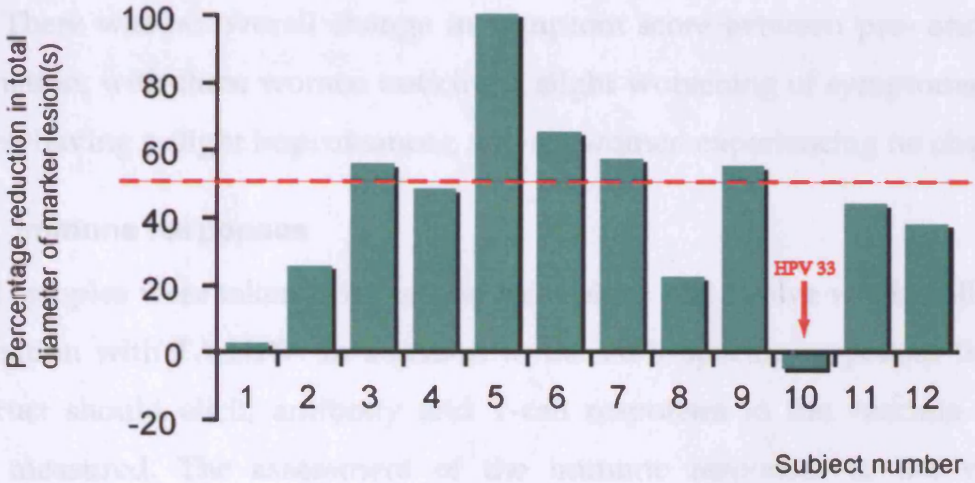


Figure 5-4. Percentage reduction in marker lesion size. Sum of widest diameter(s) at six months shown as a percentage reduction compared with the diameter pre-vaccination. Interrupted red line shows 50% reduction in size. Subject no. 10 was the only patient not positive for HPV 16.

Ten of the twelve treated women (83%) experienced some reduction in the size of the affected area over the six months after vaccination with a mean reduction in marker lesion diameter of 40% (Figure 5-4). In one woman (no. 5), there was complete clearance of the abnormal area with histological normality at three months after vaccination. In nine women, the grade of the marker lesion(s) remained unchanged, but there was a reduction of the extent of the abnormal epithelium. The size of the affected area was assessed by the longest diameter of identifiable lesion(s). In these nine individuals, the reduction in length ranged from 22 to 65% with five of the treated women showing at least 50% reduction

in total maximum lesional diameter. Two women (nos. 1 and 10) showed no reduction in the size of the affected area of vulval disease. In the 11 women in whom VIN was still present at the end of the study, the same HPV type was still detectable in the abnormal epithelium. In one patient (no. 5) whose high-grade VAIN cleared after vaccination, HPV 16 was no longer detectable in the previously abnormal area of the vagina. In one of the two patients who failed to respond to vaccination, HPV 33 was the HPV type isolated. The symptoms of VIN experienced by the patients in the study included irritation, soreness, and pain. There was no overall change in symptom score between pre- and post-vaccination, with three women noticing a slight worsening of symptoms, three women having a slight improvement, and six women experiencing no change.

5.3.4 Immune responses

Blood samples were taken prior to and four, eight and twelve weeks following vaccination with TA-HPV. In addition to the HPV-specific responses that the construct should elicit, antibody and T-cell responses to the vaccinia vector were measured. The assessment of the immune responses to the vaccine construct was performed in collaboration as described previously. The methods employed to detect such responses have been described in detail elsewhere (section 2.2.5).

5.3.4.1 Vaccine-induced immunity against the vaccinia vector.

To monitor the impact of the vaccine on the immune system, the responses to vaccinia were measured. Both antibody and T-cell responses against vaccinia were strongly increased after vaccination in almost all patients (Figure 5-5). Before vaccination, anti-vaccinia IgG was detected in nine of twelve patients (nos. 2, 3, 4, 5, 6, 8, 9, 10, and 12). Post-vaccination, IgG responses in eleven of twelve patients (not no. 8) increased approximately ten-fold. Pre-vaccination T-cell responses to the Wyeth strain vaccinia virus as measured by ELISPOT were positive in six of twelve patients (nos. 2, 4, 5, 6, 9, and 10), with frequencies ranging from 1:13,000 to 50,000. After vaccination, the IFN-γ-producing T-cell frequency to the vaccinia vector increased by approximately two-fold

(frequencies 1:2,000–10,000) compared with pre-vaccination levels in all patients, except no. 4. No positive IFN-γ responses were detected against the Vero lysate control. The increase in anti-vaccinia responses post-vaccination indicated that the patients were able to respond to the vaccinia vector component of the vaccine TA-HPV after vaccination.

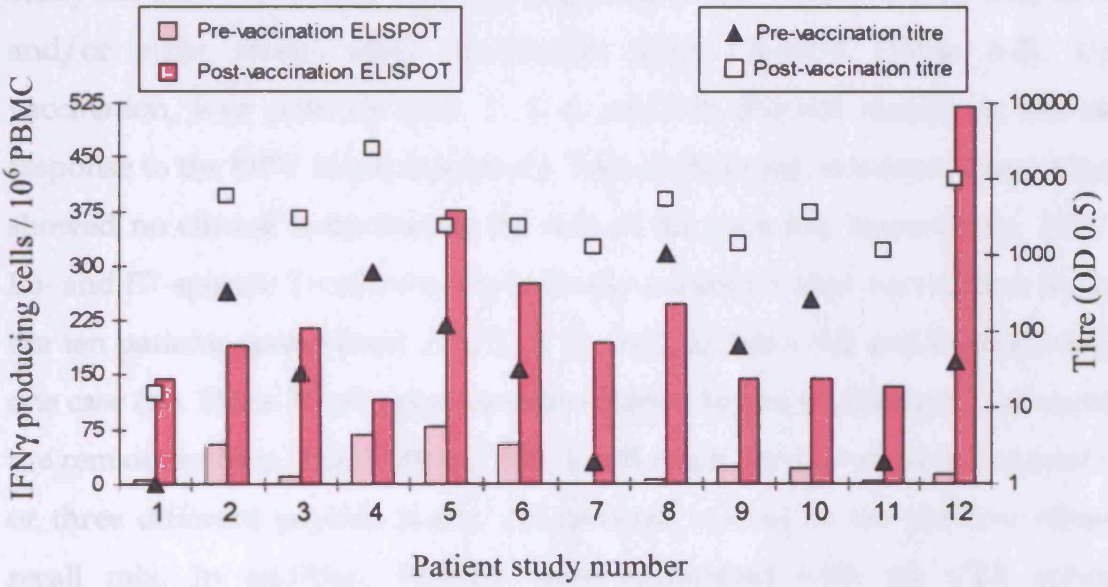


Figure 5-5. Vaccination induced immunity against the vaccinia vector. Pre- and post-vaccination peak antibody and T-cell response above background to the vaccinia vector were detected in patient serum and PBMC samples taken prior to vaccination (day 0) and at days 28, 56 and 84 post-vaccination. Peak serum antibody (IgG) responses were measured by ELISA pre-vaccination (triangle) and post-vaccination (square). Frequency of IFNγ-producing T-cells responding to vaccinia vector was measured by ELISPOT in pre-vaccination (light bar) and post-vaccination (dark bar) patient PMBC.

5.3.4.2 Pre-vaccination HPV 16-specific immune responses.

Pre-existing HPV 16-specific immunity was detected in six of the ten patients (nos. 2, 4, 5, 7, 10, and 12; Table 5-2), all of whom showed reactivity within the

HPV 16 E6 protein. Three subjects (nos. 4, 5 and 7) also demonstrated HPV 16 E7-specific T-cells.

5.3.4.3 Vaccine-induced HPV 16-specific immune responses.

TA-HPV-induced HPV 16 E6 and E7-specific T-cell immunity was analyzed by the stimulation of PBMCs with a set of overlapping peptides covering the complete amino acid sequence of the E6 and E7 proteins (section 2.2.5.3 and Figure 5-6a). From ten of twelve patients, enough material was available to study the HPV 16-specific immune response before vaccination as well as four and/or eight weeks after vaccination with TA-HPV (Table 5-2). Upon vaccination, four patients (nos. 1, 4, 6, and 10) did not mount an increased response to the HPV 16 peptide pools. Two of these patients (nos. 1 and 10) also showed no clinical reduction in the size of the lesion(s). Importantly, HPV 16 E6- and E7-specific T-cells were specifically enhanced after vaccination in six of the ten patients tested (nos. 2, 3, 5, 7, 11 and 12; Table 5-2 and Figure 5-6b). In one case (no. 3) the T-cell response was focused to one peptide pool, whereas in the remainder (nos. 2, 5, 7, 11 and 12), T-cell reactivity was detected against two or three different peptide pools. All patients reacted to the positive memory recall mix. In addition, PBMCs were stimulated with six CTL epitopes identified within HPV-16 E6/E7 and chosen for their ability to bind MHC class I molecules of HLA-A*0201-positive individuals (Kast *et al.*, 1994; Ressing *et al.*, 1995). Of the ten patients typed as HLA-A2 (Table 5-1), one patient (no. 3) responded weakly to peptide HPV 16 $E6_{80-88}$ (T-cell frequency 1/50,000). This response was already present before vaccination and the single injection of TA-HPV failed to enhance this reactivity. Two patients (nos. 7 and 12) showed an enhanced production of IFN-γ in response to the whole HPV 16 E6 and E7 protein as expressed in TA-CIN after vaccination with TA-HPV. No other patient reacted to TA-CIN either pre- or post-vaccination (Table 5-3).

Patient	Date (weeks)	E6 pool 1	E6 pool 2	E6 pool 3	E6 pool 4	E7 pool 1	E7 Pool 2	MRM
1	0	<1	<1	1	<1	<1	<1	23
	4	1	1	4	<1	1	2	49
	8	NA	NA	NA	NA	NA	NA	NA
2	0	<1	26	<1	<1	<1	<1	100
	4	1	1	3	**5**	<1	1	100
	8	<1	26	<1	**27**	7	<1	100
3	0	<1	2	<1	<1	<1	<1	50
	4	<1	**57**	<1	<1	2	<1	46
	8	5	**35**	<1	<1	<1	<1	57
4	0	<1	6	<1	<1	<1	6	124
	4	NA	NA	NA	NA	NA	NA	NA
	8	<1	<1	<1	<1	<1	<1	103
5	0	<1	5	1	<1	10	3	119
	4	<1	**18**	**9**	<1	10	**14**	87
	8	1	**86**	**15**	<1	1	**33**	85
6	0	<1	<1	<1	<1	<1	<1	61
	4	<1	<1	<1	<1	<1	<1	79
	8	<1	1	<1	<1	4	<1	63
7	0	4	21	7	<1	5	<1	114
	4	**23**	**84**	1	1	**44**	<1	106
	8	**15**	**66**	<1	**7**	**24**	<1	91
10	0	<1	<1	1	15	1	<1	51
	4	<1	0	2	7	1	1	108
	8	<1	<1	<1	6	<1	<1	79
11	0	1	<1	1	<1	3	<1	63
	4	<1	<1	<1	<1	**9**	<1	93
	8	<1	<1	<1	**16**	<1	<1	54
12	0	4	5	13	2	4	4	104
	4	**84**	1	**42**	<1	<1	<1	58
	8	**39**	<1	17	<1	<1	<1	71

Table 5-2. TA-HPV enhanced HPV16 E6- and E7-specific immune responses. PBMC of 10 patients were available for complete analysis of the HPV16 E6- and E7-specific T-cell response by IFNγ– ELISPOT assay. Specific spots per 50,000 PBMC are indicated. Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots of experimental wells. Indicated in bold are those peptide pools (and number of spots) to which specific T-cell frequencies were ≥ 1/10,000 PBMC and at least 3-fold the pre-vaccination response, because these are considered to be vaccine enhanced. NA, not available for analysis. <1, no specific response to E6 or E7. MRM, memory recall mix used as a positive control.

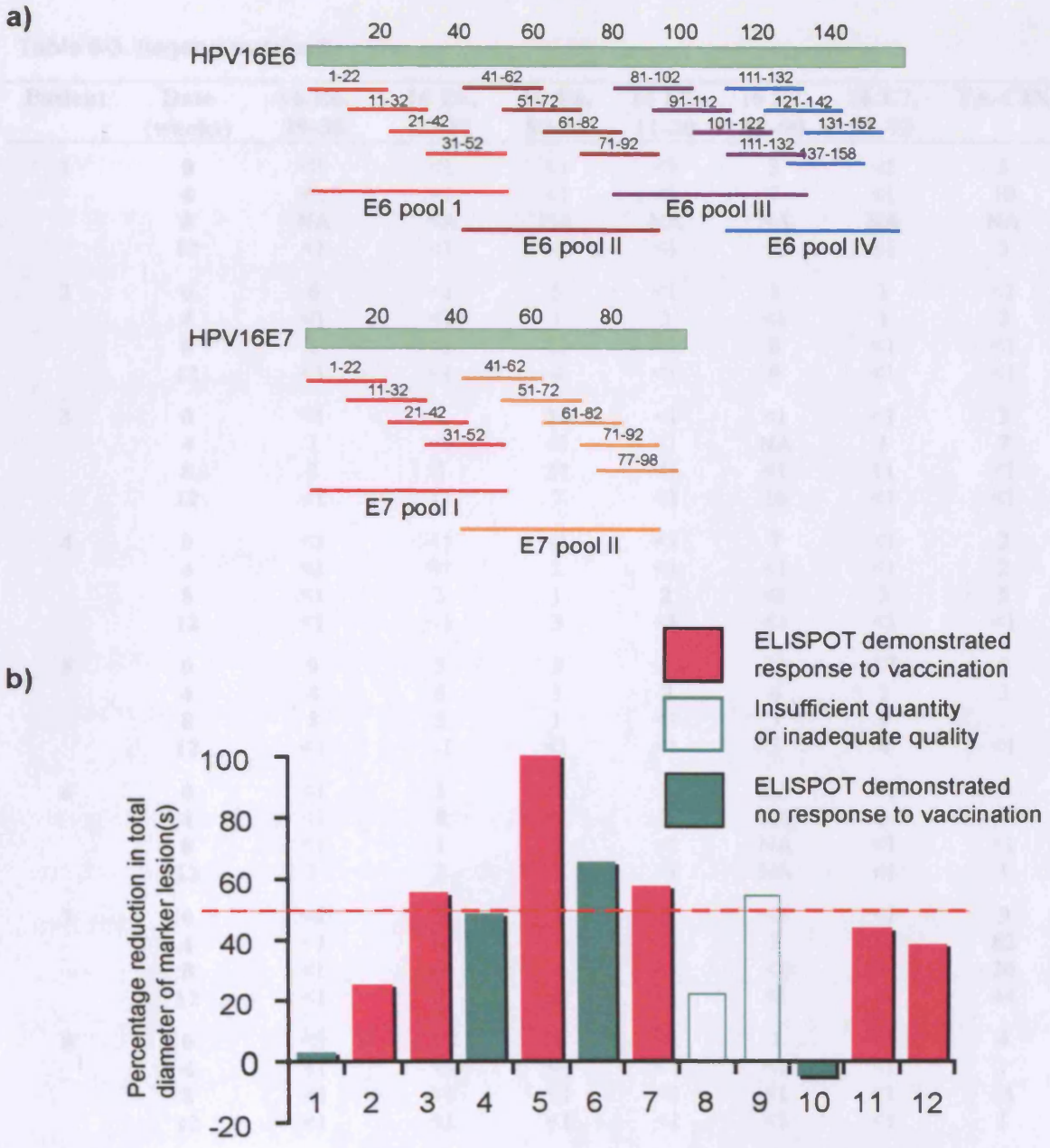


Figure 5-6. Vaccine-induced immunity and clinical responses

a) Groups of overlapping peptides used in IFN-γ ELISPOT to assess HPV-specific T-cell immunity. Numbers indicate beginning and end of peptide.

b) Percentage reduction in marker lesion size correlated with HPV-specific T-cell immunity. Sum of widest diameter(s) at six months shown as a percentage reduction compared with the diameter pre-vaccination. Interrupted red line shows 50% reduction in size. Pink bars indicate subjects who showed vaccine induced T-cell response as assessed by ELISPOT.

Table 5-3. (legend overleaf)

Patient	Date (weeks)	16 E6, 29-38	16 E6, 59-67	16 E6, 80-88	16 E7, 11-20	16 E7, 82-90	16 E7, 86-93	TA-CIN
1	0	<1	<1	<1	<1	3	<1	3
	4	<1	<1	<1	<1	7	<1	10
	8	NA	NA	NA	NA	NA	NA	NA
	12	<1	<1	<1	<1	<1	<1	3
2	0	6	<1	5	<1	3	1	<1
	4	<1	<1	1	3	<1	1	2
	8	1	<1	13	<1	6	<1	<1
	12	<1	<1	4	<1	9	<1	<1
3	0	<1	3	**24**	<1	<1	<1	3
	4	1	<1	<1	<1	NA	1	7
	8	3	3	**27**	<1	<1	11	<1
	12	<1	<1	7	<1	16	<1	<1
4	0	<1	<1	<1	<1	7	<1	2
	4	<1	<1	1	<1	<1	<1	2
	8	<1	3	1	2	<1	3	5
	12	<1	<1	3	<1	<1	<1	<1
5	0	9	5	5	2	11	17	9
	4	4	6	3	2	6	2	3
	8	3	5	1	<1	7	9	1
	12	<1	<1	<1	<1	5	4	<1
6	0	<1	3	<1	2	NA	<1	5
	4	<1	8	<1	2	NA	3	6
	8	<1	1	3	<1	NA	<1	<1
	12	1	3	3	<1	NA	<1	1
7	0	<1	3	1	2	<1	<1	9
	4	<1	<1	3	<1	2	14	**82**
	8	<1	<1	4	<1	<1	6	**30**
	12	<1	1	4	<1	<1	12	**44**
8	0	<1	<1	3	<1	1	<1	4
	4	<1	<1	<1	<1	<1	<1	1
	8	<1	<1	<1	<1	<1	<1	<1
	12	<1	<1	<1	<1	<1	<1	1
9	0	<1	2	2	<1	7	<1	2
	4	<1	2	<1	<1	5	3	9
	8	<1	<1	<1	1	4	<1	<1
	12	<1	<1	<1	<1	1	<1	<1
10	0	<1	<1	<1	<1	<1	<1	7
	4	<1	<1	<1	<1	<1	<1	<1
	8	<1	<1	<1	<1	<1	<1	1
	12	<1	<1	<1	1	<1	<1	2
11	0	<1	<1	<1	<1	<1	<1	<1
	4	<1	1	1	<1	<1	5	1
	8	1	1	5	1	<1	1	7
	12	<1	<1	<1	<1	<1	<1	4
12	0	<1	<1	<1	<1	<1	<1	13
	4	<1	<1	3	<1	<1	<1	**69**
	8	2	<1	3	<1	<1	1	**53**
	12	<1	<1	<1	<1	<1	<1	16

Table 5-3. Patient immune responses to HPV 16 E6 and E7 peptides and TA-CIN protein

PBMC isolated from whole blood were cultured overnight with peptides to elicit IFNγ release as detected by ELISPOT assay. In addition, whole HPV16 E6 and E7 protein (TA-CIN) was used to stimulate harvested PBMC. Specific spots per 2 x 10^5 PBMC are shown. Specific spots were calculated by subtracting the mean number of spots from 3 wells of the medium control from the mean number of spots of experimental wells. Values in bold are those over 20 and are taken as a positive response. NA, not available for analysis. <1, no specific response to peptide or protein.

5.4 Discussion

This chapter presents the results of a phase II clinical trial to assess the safety, immunogenicity and efficacy of a recombinant vaccinia vector expressing HPV 16 and HPV 18 E6 and E7 proteins (TA-HPV) in the treatment of AGIN. In this study of twelve women with high-grade VIN and VAIN, no adverse effects of the vaccination were found. In general, the patients felt well without definite evidence of pyrexia. The vaccination site was tender and uncomfortable in all women with duration of discomfort ranging between one and thirteen days, which was maximal approximately one week after vaccination. The vaccination site was dressed for a mean duration of 25 days (range, 15–32 days) and was acceptable to all patients. Vulval symptoms were not significantly improved by vaccination. It may be that an alternative reporting system, *e.g.*, visual analogue scale might prove more sensitive in detecting symptom change. No progression of disease was observed in any of the twelve women during the 6 months after vaccination. In eleven women, no change in disease grade occurred, but the reduction in size of the measured regions of AGIN seen in ten of the twelve women was promising. In five women, the affected areas were reduced by ≥50% of the original longest diameter, and in one patient, the lesion cleared completely. The lack of a placebo group in this study raises the possibility that this response might not be attributable to the vaccination. However,

spontaneous resolution of high-grade disease (section 1.1.3.1) appears to be infrequent and confined to a relatively discrete population of women whose characteristics are not shared by the current cohort. Furthermore, this response rate is similar to that of a recently published trial using the same construct in which eight of eighteen patients with high-grade VIN showed at least a 50% reduction in marker lesion size (Davidson *et al.*, 2003a). An effective treatment that would compare favorably with the most common form of treatment, excisional surgery, would be one in which complete clearance occurred and in which recurrence was rare. The responses seen in this small group of women with diverse duration of disease and prior treatment do not achieve this ideal, although as yet, it is impossible to know if the longer-term course of the disease has been influenced by the stimulation of anti-HPV immunity. Interestingly, the patient who showed a complete response (no. 5) also showed resolution of concurrent CIN (grade I). Although spontaneous resolution of low-grade cervical disease is common (Duggan *et al.*, 1998), persistence of her clinical response to twenty-four months after vaccination is extremely encouraging, especially given her prolonged (~10 year) pre-trial history of relapsing cervical and latterly vaginal disease. A reduction in lesion size also enabled several subjects to undergo relatively minor excisional treatment at the conclusion of the study, compared with the radical approach that might have been used to deal with their disease at enrolment.

The development of a cell-mediated immune response appears to be crucial to the control and clearance of HPV-related disease (section 1.2.2). Indeed, the patient with idiopathic CD4+ deficiency who was excluded from vaccination during this work suffers with the most extensive multifocal AGIN that the author has encountered. However, the precise nature of the immune response to HPV is still poorly understood. It has earlier been reported that women with HPV 16 positive cervical disease may have T-cell responses to HPV-16 E7 peptides (Kadish *et al.*, 1994; Boursnell *et al.*, 1996; Muderspach *et al.*, 2000; van der Burg *et al.*, 2001b; Kadish *et al.*, 2002). In our patients, we found measurable immune responses to at least one HPV 16 E6- or E7-peptide-pool in six of the

analyzed women (60%) pre-vaccination (Table 5-2). Immunoreactivity was found against all HPV 16 E6- and E7-peptide-pools, but with slightly greater frequency or strength to peptides covering the central E6 region from amino acid 41-132. Others have suggested that T-cell responses to HPV-16 E7 peptides may be reduced in women with invasive cancer (de Gruijl *et al.*, 1998; van der Burg *et al.*, 2001b) but relatively increased in persistent and especially high-grade cervical dysplasia (de Gruijl *et al.*, 1998). The low level of HPV 16 E7-stimulated IFN-γ production by T-cells from this cohort of patients with VIN 3 could reflect an apparent suppression of immunity in chronic HPV disease or tolerance to 16 E7. The previously reported preponderance of immunoreactivity to -COOH terminal HPV-16 E7 peptides in patients with CIN (Luxton *et al.*, 1996; de Gruijl *et al.*, 1998) or central E7 peptides in high-grade CIN and cervical cancer (van der Burg *et al.*, 2001b) was not seen in these patients with VIN/VAIN. A systemic immune response upon vaccination was detected by ELISPOT assays to pooled peptides in six of the ten patient samples available for testing (Table 5-2 and Figure 5-6). All these six subjects showed a clinical response. In four women (40%), there was no significant increase in the number of lymphocytes producing IFN-γ after exposure to viral peptides following vaccination compared with the pre-vaccination responses. Two of these four women had no clinical response either, but the remaining two patients showed a significant (≥50%) reduction in marker lesion diameter. There was no strong correlation between the clinical change and the observed *in vitro* T-cell response. It is possible, however, that the detection of systemic immunity to HPV-16 E6 and E7 peptides after vaccination may not truly reflect the intensity of the immune response at the site of the disease. Nevertheless, the observed T-cell responses in six of eight clinical responders remain remarkable.

Other studies of vaccination as therapy for HR-HPV-associated anogenital disease have been reported recently. The use of DNA (Klencke *et al.*, 2002), peptides (Steller *et al.*, 1998; van Driel *et al.*, 1999; Muderspach *et al.*, 2000; Ressing *et al.*, 2000; van der Burg *et al.*, 2001b), protein (Goldstone *et al.*, 2000), recombinant virus (Borysiewicz *et al.*, 1996; Davidson *et al.*, 2003a), dendritic

cells (Santin *et al.*, 1999) and virus-like particles (Rudolf *et al.*, 1999) have all been shown to induce an HPV-specific immune response. The development of a cytotoxic T-cell response probably in combination with a T-helper cell response is likely to produce the best immune attack on virally infected neoplastic cells. HLA class I-restricted HPV-16 E6 and E7 peptides most likely to produce a cytotoxic T-cell response have been defined (Kast *et al.*, 1994; Ressing *et al.*, 1995). CTL responses were assessed in the current work using the ELISPOT technique described in detail in section 2.2.5.3. The use of well-defined minimal CTL peptide-epitopes in this fashion elicits strong responses from CD8+ T-cells, whereas CD4+ T-cell reactivity is usually weak or non-detectable because of the short incubation time used (van der Burg *et al.*, 2001b). In our patients, there was no stimulation of T-cell responses to these peptides after vaccination (Table 5-3). However, the use of a restricted set of peptides to analyze CTL reactivity may be inadequate to permit the detection of all CTL responses. In support of this testing strategy, it should be noted that eleven of twelve patients were HPV 16-positive and that in common with the findings of previous work (Davidson *et al.*, 2003b), HLA-A2 was over-represented in these patients with high-grade VIN/VAIN (10/12 patients). Other studies of vaccination in humans using these selected peptides have suggested that patients with CIN, cervical cancer, and vaginal cancer are able to mount a $CD8^+$ T-cell response to the peptides (Steller *et al.*, 1998; Muderspach *et al.*, 2000), although in one study, the lack of a peptide-specific cytotoxic T-cell response was similar to the results presented here (Ressing *et al.*, 2000).

In ten patients, T-cell responses were further assessed by ELISPOT using pools of 15-mer peptides spanning HPV16 E6 and E7 (Figure 5-6 and section 2.2.5.3). In two patients, PBMC samples were either insufficient or had deteriorated during storage such that further immune testing was impossible (patients 8 and 9). In contrast to the lack of response seen using the defined CTL epitopes, an increased response to one or more peptide-pools following vaccination was seen in six of ten women tested. Work from our collaborators in Leiden suggests that the use of these longer peptides, which must be taken up,

processed and presented by APCs, together with the long incubation period of four days used in the ELISPOT, highly favours the response of CD4+ T-cells (van der Burg *et al.*, 2001b; de Jong *et al.*, 2002; Welters *et al.*, 2003). These responses have not been previously tested in other vaccination trials in humans.

The effect of vaccination on HPV infection and HPV-associated neoplastic disease is less clear. In CIN, the development of HPV16 E7-specific CMI has recently been associated with both disease regression and the resolution of viral infection (Kadish *et al.*, 2002). Following vaccination with TA-HPV in the current study, the same HPV type was still detectable by PCR in abnormal epithelium, even in those patients with a significant (≥50%) decrease in marker lesion size. However, in the one patient who cleared her disease (no. 5) HPV 16 could no longer be found in the previously abnormal area of the vagina. Quantitative PCR to assess changes in viral load following vaccination may have been more informative than the qualitative PCR techniques employed to assess HPV status. Davidson and colleagues used real time PCR technology to assess viral load before and following vaccination with TA-HPV in high-grade VIN (Davidson *et al.*, 2003a). Viral load was found to decrease following vaccination in six of eight patients (75%) deemed to have a clinical response (≥50% decrease in marker lesion diameter). However, a decrease in viral load was also demonstrated in six of the ten (60%) remaining patients who failed to demonstrate a clinical response. It is therefore difficult to associate a reduction in viral load with clinical improvement of disease.

Only small numbers of patients with HR-HPV dysplasia or carcinoma have received vaccination as therapy, and as yet, dramatic clinical responses have not occurred. Borysiewicz *et al.* (1996) reported the use of TA-HPV in eight women with therapy-unresponsive cervical cancer, one of whose disease remitted. Although the vaccine has been used as adjuvant therapy in two small trials of women with CIN 3 before laser therapy and in women with early-stage cervical cancer before surgery, the possible effect on the disease was not evaluated (Adams *et al.*, 2001). HPV peptides have been used as therapeutic vaccines in several small studies. In one of these, eleven women with cervical cancer and

one woman with vaginal cancer, all of whom were of the HLA-A2 genotype, received immunotherapy with the HPV 16 $E7_{86-93}$ peptide (Steller *et al.*, 1998). None of these patients showed a clinical response to vaccination, although two individuals demonstrated induction of specific and persistent CTL responses. As has already been described, a second trial of TA-HPV used in the treatment of high-grade VIN has recently been published (Davidson *et al.*, 2003a). The authors of this study found similar clinical response rates to those described in this chapter. Davidson *et al.* used HLA-A2 restricted peptides in an ELISPOT to assess HPV-specific T-cell responses. Four of seven HLA-A2 positive responders showed an increased CTL response following vaccination. In two of these cases the response was a novel one rather than an increase in existing T-cell reactivity.

Another HLA-A2-restricted peptide $E7_{12-20}$ has been tested as a therapeutic vaccine in eighteen women with HPV 16-positive high-grade intraepithelial neoplasia of the cervix (n =16) or vulva (n=2) (Muderspach *et al.*, 2000). Seven of these women also received the same HPV 16 E7 peptide, $E7_{86-93}$ used by Steller and colleagues (Steller *et al.*, 1998). There was observed improvement of disease in terms of partial (6/18) or complete (3/18) clearance of affected areas with improvement only occurring in women with CIN. Ten of sixteen women tested had induction of E7 immunoreactivity as assessed by IFN-γ release and chromium release assays. The combination of these two HPV 16 E7 peptides, $E7_{11-20}$ and $E7_{86-93}$, has also been tested as therapy in nineteen women with late-stage HPV 16-positive cervical cancer who were HLA-A*0201-positive (van Driel *et al.*, 1999). The vaccination was well tolerated but produced no clear effect on disease course. A third type of vaccine tested in human disease has recently been reported (Klencke *et al.*, 2002). Plasmid DNA encoding the amino acids 83–95 of HPV 16 E7 was used as a vaccine in patients with AIN. Three of ninteen treated individuals were found to have changed from high- to low-grade AIN, whereas in the remainder, the disease did not change.

Ideally, therapeutic vaccination for HPV-associated AGIN would have an efficacy that induces complete disease regression without recurrence. To

produce such an effect, the immune response should be readily induced and maintained and must be able to produce a potent cytotoxic or delayed type hypersensitivity response against epithelial cells harboring the viral DNA. The development of a therapeutic vaccine to combat this virally driven disease is still some way from producing these aims. However, when interpreted with the results of other studies of therapeutic vaccination, the work presented in this thesis suggest that stimulation of the immune response in HR-HPV-infected individuals may be able to produce at least a partial effect on neoplastic disease. Improvements in vaccine design and delivery as well as techniques to optimize immune responses within the diseased epithelium may ensure that therapeutic vaccination becomes an effective treatment choice for HPV-associated AGIN.

Chapter 6 Summary, conclusions and future directions

The diagnosis of VIN does not inevitably lead to the development of vulval SCC. However, a review of the available evidence would suggest that, at least for some individuals, the condition is pre-malignant in nature (section 1.1.3). Despite the potential for investigation and intervention at this earlier stage, the viral, genetic and immunological events that occur during vulval oncogenesis have been poorly understood. Previous studies have concentrated on defining changes associated with SCC lesions, the end-point of vulval oncogenesis. In contrast, the current work has focussed on the potentially pre-invasive intraepithelial lesion and provides insight into both the pathophysiology and potential immunotherapy of HPV-related vulval neoplasia. It is hoped that a better understanding of VIN will allow for more effective, individualised treatment of women who suffer with this often distressing condition.

6.1 VIN shows a strong association with HR-HPV infection

The cohort of women involved in the current work appears to be typical of those suffering with VIN and vulval SCC. Many women with VIN suffer recurrent or relapsing disease. The finding that two-thirds of the patients with VIN were under the age of fifty years would appear to support the stance that an increasing proportion of women with VIN are young (Jones *et al.*, 1994; Herod *et al.*, 1996; Joura *et al.*, 2000). The strong association between HPV infection and high-grade VIN was confirmed in the current work. Unfortunately, the low incidence of HPV-negative disease has limited the ability of this work to establish differences between individuals with HPV-positive and HPV-negative VIN and therefore this aim has not been achieved. However, the high incidence (>90%) of HPV infection in VIN found both in this work and by others (van Beurden *et al.*, 1995; van Beurden *et al.*, 1998a) may indicate that such differences are unlikely to be of clinical relevance and it may therefore be more productive to focus future efforts on defining different clinico-pathological subgroups within the cohort of patients with HPV-positive

VIN. Rather than making the absolute division between HPV-positive and HPV-negative cases, it may be more informative to consider viral load as assessed by real-time PCR. Indeed, for cervical disease, viral load has been proposed as a predictor for the development of future HSIL (Josefsson *et al.*, 2000; Ylitalo *et al.*, 2000) and copy number of HPV 16 DNA and RNA may correlate directly with the grade of SIL as assessed by cervical cytology (Wang-Johanning *et al.*, 2002). The detection of multiple HPV infections was limited by the typing methodology chosen (section 1.1.4.1.1). However, in keeping with the findings of others, there was a preponderance of HPV 16 infection in cases of VIN (~94%; section 3.3.1). This is a HR-HPV type with a proven role in the development of carcinoma of the cervix (Bosch *et al.*, 2002). The strong association between VIN and HR-HPV provides a plausible mechanism for oncogenesis in the vulva and lends support to those who, like the author, feel that VIN is a potentially pre-malignant condition (section 1.1.3). The data are not however strong enough to claim a causal relationship between HPV and vulval neoplasia.

6.2 VIN is associated with transcriptionally active HR-HPV

RNA ISH using digoxigenin-labelled riboprobes was successfully used to demonstrate transcriptionally active HPV 16 in VIN lesions as well as both vulval SCC and histologically 'normal' vulval skin (section 3.3.3). The pattern of viral gene transcription did not allow the selection of a potentially high-risk group of patients with VIN and this aim was not therefore acheived. Specimens tended to be either 'positive' or 'negative' for most of the transcripts studied. The various methodological issues that may explain this finding have been discussed at length in Chapter 3. The use of more sensitive methods such as radio-labelled riboprobes or tyramide signal amplification for future studies may allow differentiation between sub-groups of VIN lesions in terms of differentiating patterns of viral transcription.

6.3 Viral integration appears to occur early in the development of HPV-related vulval malignancy

Transcriptionally active HPV was demonstrated in histologically normal vulval skin before the onset of dysplasia. HPV may therefore be important in vulval oncogenesis at an early stage. The effects of cellular factors and host responses on viral transcription may be crucial in the containment of such infections. Viral integration has been proposed as a method by which HPV transcription may become de-regulated. Following integraton, the resultant preponderance of transcripts from the high-risk ORFs increases the capacity of the HPV to produce immortalisation and malignancy (Schwarz *et al.*, 1985; Cripe *et al.*, 1987; Romanczuk *et al.*, 1992). Integration, as represented by the loss of the E2 signal at PCR, was demonstrated in approximately half the HPV-positive VIN samples studied (section 3.3.1). The limitations of this methodology to assess integration have been described in Chapter 3 and it is likely that this method will underestimate the true extent of viral integration in VIN. It would appear that, like viral transcription, HPV integration occurs at an early rather than late stage in the development of HPV-related vulval malignancy. This hypothesis would be in keeping with recent *in vitro* work using the W12 cell line, in which viral integration was shown to precede the development of high-level chromosomal instability (Pett *et al.*, 2004). Given the relatively high frequency of HPV integration in VIN lesions, the detection of integrants may prove to be of limited value in the defining subsets of VIN at particular risk of progression to carcinoma. The use of techniques such as APOT (Klaes *et al.*, 1999) to detect transcriptionally active integrants, might be of more prognostic significance than simply demonstrating viral integration within a specimen.

6.4 *Chromosomal alterations in VIN occur in a non-random fashion and the nature of CNIs observed may contribute to the progression of intra-epithelial lesions*

The consequence of HR-HPV infection with viral transcription and integration would appear to be the production of an environment that favours the acquisition of genetic damage. Whilst a limited number of abnormalities were seen in low-grade disease, the use of CGH to screen VIN biopsies has revealed high-grade VIN (VIN 2-3) to be a genetically unstable lesion, with CNIs commonplace (section 4.3). There is much uncertainty regarding the diagnosis and clinical significance of low-grade VIN. Certainly, where CNIs are concerned, this thesis provides a genetic basis upon which to consider moving from a three-tier to a two-tier classification system (section 4.3.3.4), especially with the improvements in pathological classification that such a system affords (Preti *et al.*, 2000). Of the few abnormalities seen in low-grade lesions, gain of the telomeric end of 1p may confer a selection advantage that assists the progression to high-grade VIN. The nature of the common CNIs observed within the high-grade VIN lesions was comparable to those seen in high-grade CIN (section 4.3.3.1). In particular, several gains that have been proposed as potential markers of invasive potential in cervical neoplasia were also demonstrated in a subset of high-grade VINs. Gain of 3q was seen in eight individuals with high-grade VIN and associated with loss of 3p suggestive of isochromosome formation in five cases. CNIs affecting this region are thought to be of particular relevance in the formation of HPV-positive SCCs of the genital tract (section 4.4). Interestingly, two of the subjects in whom gain of 3q was demonstrated within an area of VIN had associated invasive lesions. The implications of the high-level gain of 5p found in one case of high-grade VIN have been discussed and the localised region of CN gain affecting 11q lends support to the possible role of oncogenes such as CCND1 and EMS1 in vulval oncogenesis (section 4.4).

The pattern of CNIs detected by CGH suggests that the acquisition of CNIs affecting chromosome 8 may provide a selection advantage that facilitates the progression of high-grade VIN lesions to SCCs. Whilst loss of 8p and gain of 8q were significantly more common in SCC, both were present in high-grade VIN lesions. The two cases exhibiting CN loss on 8p (study no's 271 and 283) were both thought to be at particular risk of progression, one patient being immunocompromised following a renal transplant and the other because there was an invasive lesion adjacent to the area of VIN 3. CGH data from the cervix have also demonstrated CN loss in this region for both SCCs (Heselmeyer *et al.*, 1997b) and high-grade CIN (Kirchhoff *et al.*, 2001). Analysis of oral SCCs suggests that the region contains at least two TSGs (Partridge *et al.*, 1999).

The CNIs highlighted by this extensive CGH study of VIN should ideally be confirmed by interphase FISH. Microsatellite markers may be used to localise more precisely potential candidate TSGs. The functional consequence of the CNIs identified could be established by expression profiling. High throughput techniques such as the use of array technology could facilitate this future work.

6.5 The induction of CMI in individuals with VIN is both desirable and feasible

Progression from high-grade VIN 3 to invasive SCC is not inevitable, despite the frequent finding of oncogenic HPV infection and marked CNIs. It is likely that the host response to virally infected, dysplastic cells is crucial for disease containment. HLA polymorphism has been repeatedly linked with either increased or decreased susceptibility to cervical neoplasia (section 1.2.2.4). We have recently demonstrated similar associations with HLA polymorphisms in VIN (Davidson *et al.*, 2003b). However, individuals are not reliant solely on innate immunity. The fact that high-grade VIN is associated with a high frequency of transcriptionally active HPV 16 infection (Haefner *et al.*, 1995; Hørding *et al.*, 1995; van Beurden *et al.*, 1995; Madeleine *et al.*, 1997; van Beurden *et al.*, 1998b) provides a welcome opportunity to influence the natural history of the disease with immunotherapy. In the current work HPV was isolated from

95% of cases of VIN. Effective prophylactic vaccination against HPV infection by the induction of HPV-specific antibodies is already a reality (Koutsky *et al.*, 2002). The limited number of HPV types implicated in VIN would make the choice of viral epitopes for such a vaccine much easier than for cervical neoplasia. A prophylactic vaccine that induced effective sero-immunity against HPV 16 alone could prevent at least 85% of cases of HPV-related VIN (Haefner *et al.*, 1995; Hørding *et al.*, 1995; van Beurden *et al.*, 1995; van Beurden *et al.*, 1998a; Rosenthal *et al.*, 2001). By increasing coverage with a multivalent vaccine effective against types 16, 18, 31 and 33 almost all cases of HPV-related VIN could theoretically be prevented. The majority of infections are acquired sexually, providing a window of opportunity for vaccination in childhood, before the onset of sexual activity. However, the vaccination of children is already an area of much controversy and there are practical, moral and ethical concerns about mass vaccination for a 'sexually-transmitted' disease in this age group. Furthermore, vertical transmission of high-risk genital HPV types does occur (Rice *et al.*, 1999; Rice *et al.*, 2000; Smith *et al.*, 2004) and this research has demonstrated infection with other HPV-types such as the intermediate-risk type 56. Therefore, prophylactic vaccination is unlikely to provide effective prevention against VIN in all individuals and patients will still present with established disease. Whilst seroconversion alone in this group is unlikely to cause complete disease regression, there may still be some advantage in inducing sero-immunity in women with pre-existing HPV infection. It is possible that the induction of mucosal antibodies may limit the spread of HPV to more susceptible areas of the lower genital tract e.g. the transformation zone. In addition, vaccination to induce humoral immunity may help to limit cross-infection between sexual partners.

The increased risk of disease progression and high incidence of multicentric disease in immunocompromised individuals (Leckie *et al.*, 1977; Conley *et al.*, 2002) would support the role of the host cellular immune response in limiting the effects HPV-related genital intraepithelial neoplasia. The work presented in this thesis demonstrates that vaccination of women with high-grade VIN using

a recombinant vaccinia vaccine expressing HR-HPV E6 and E7 is both practical and safe. Environmental containment of the genetically modified vaccinia was achieved with a simple bio-occlusive dressing. No serious adverse events were recorded and the local reaction to vaccination only limited activity in two of the twelve patients vaccinated (section 5.3.2).

6.6 The induction of cellular immune responses to non-structural HPV 16 proteins was associated with clinical disease regression

Following vaccination with TA-HPV, there was no effect on viral transcription as assessed by mRNA ISH. However, a number of the subjects showed a partial clinical response, with one individual undergoing complete regression of her longstanding intraepithelial disease. The limitations in the sensitivity of the ISH methodology used have been highlighted previously. A quantitative assessment of viral copy number e.g. using real-time PCR technology may demonstrate differences in viral load (either DNA or RNA) following vaccination. In a similar trial, DNA copy number was reduced in 12/18 patients with VIN following vaccination with TA-HPV although no correlation between this reduction and disease regression was demonstrated (Davidson *et al.*, 2003a).

The current work is one of the first studies to demonstrate that the development of CMI to non-structural HPV proteins can be associated with disease regression and this hypothesis can therefore be accepted (sections 5.3.3 and 5.3.4). The findings have been reproduced by other investigators (Davidson *et al.*, 2003a). Previous studies have focussed on individuals with advanced stage carcinoma. Such patients commonly have deletions and mutations affecting genes involved with antigen processing and presentation (Brady *et al.*, 2000; Evans *et al.*, 2001) providing a potential mechanism for viral immune evasion. A potential therapeutic effect in earlier stage disease may therefore be missed by such studies. Despite evidence from animal models suggesting that HR-HPV E6 and E7 provoke weak natural immune responses (reviewed by Frazer, 2004),

these oncoproteins remain an attractive target for therapeutic vaccination in humans. Both these ORFs are consistently preserved following integration, itself a key step in oncogenesis and their transcription may be enhanced by this process (section 1.2.1.5). Certainly, integration appears to be a frequent and often early event in VIN and the choice of vaccination target would have to reflect this finding. In addition, naturally occurring immunity to the HR-HPV oncoproteins does occur and may confer protection against genital carcinoma. T-helper responses to HPV E6 are found in large numbers of healthy individuals (Welters *et al.*, 2003) and are frequently absent or defective in cervical cancer patients (de Jong *et al.*, 2002). Delayed-type hypersensitivity to HPV 16 E7 is associated with clinical and cytological regression of HPV-related CIN (Höpfl *et al.*, 2000). The present work confirms that immune responses can be induced to HPV 16 E6 and E7 and importantly, that vaccination may be therapeutic. However, the goal of an effective therapeutic vaccine for HR-HPV is still some way off. Most studies have concentrated on the detection of an immune response in the peripheral circulation. Although a response at this site is of interest, from a biological viewpoint, the response at the tumour site is certainly more relevant. The limited data available suggest that the frequency of induced CTLs is higher in the local tissues and loco-regional lymph nodes (Evans *et al.*, 1997). Such tissue specificity may well be determined early on at the time of antigen presentation (Mora *et al.*, 2003) and the route of vaccine delivery is therefore of paramount importance. The problem of achieving tissue specificity other than by local administration will need to be overcome. The use of topical immunomodulators such as Imiquimod could assist in the recruitment of systemic, vaccine-induced CTLs to the tumour site.

Combination vaccinations could potentially enhance the generation of HPV-specific CTL responses. We have recently participated in a trial of heterologous prime-boost immunisation strategy. Pre-clinical studies suggested that the use of peptide-priming with a fusion protein (HPV 16 L2E6E7) followed by boosting with TA-HPV (recombinant vaccinia virus encoding HPV 16/18 E6 and E7) was superior to the use of either agent in isolation (van der Burg *et al.*,

2001a). However, whilst this regime induced CMI, the advantages seen in animal models were not replicated in humans and no clear relationship between the induction of immunity and clinical outcome was demonstrated (Smyth *et al.*, 2004). Ensuring effective antigen presentation by delivering peptides packaged in antigen-presenting dendritic cells may overcome some of the methods of immune escape that are evident in HPV-related genital neoplasia. Whilst efforts are focussing on producing ever-greater levels of T-cell responses, the nature of the induced response is crucial. Multiple dose regimens and adjuvants have been used to produce enhanced CMI in peptide vaccination. However, the responses generated tend to favour the development of a Th2 rather than the desired CTL Th1 response (for review see Frazer, 2004). HPV appears well placed to avoid the host immune response by a combination of stealth and immune evasion. Choosing both the route of vaccination and ensuring that antigen presentation occurs with the correct sequence of events will be pivotal to generating effective responses at the tumour site.

6.7 *Implications for clinical care and future clinical trials*

The strong association with transcriptionally active HR-HPV types and the genetically unstable nature of the lesions studied suggests that VIN has the potential to progress to carcinoma. Although limited data exist regarding the natural history of untreated VIN lesions, these observations would suggest that caution is required when conservative management protocols are adopted and when new unproven treatments are introduced. During clinical trials, it would seem prudent to include variables such as HPV status and CNIs in the evaluation of therapeutic effect.

The use of CGH to study vulval neoplasia has demonstrated frequent CNIs, many of which are similar to those found in CIN. The marked difference in the pattern and frequency of CNIs between low- and high-grade VIN lesions would favour the adoption of a two tier classification system, analgous to the Bethseda system currently in use for cervical pre-malignancy (Solomon *et al.*, 2002). Such a system would appear biologically plausible and might well help to minimise

differences between studies that are currently attributable to variance in pathological interpretation (Preti *et al.*, 2000). The similarities demonstrated between VIN and CIN suggest that novel therapeutic options defined for VIN are likely to be applicable to cervical disease. Vaccination would appear to be safe and practical and the demonstration of a therapeutic effect in association with the induction of HPV-specific CMI is extremely encouraging to those who are trying to find effective new therapies for HPV-related AGIN. Several potential strategies to improve these responses have been highlighted in the previous section. Small phase II studies are invaluable in the early phase of vaccine development. However, the relative infrequency of VIN means that only multicentre studies are likely to generate data of sufficient power to guide clinicians in their management. It is hoped that by co-operation between investigators in this field, more rapid progress in the understanding and treatment of this challenging condition will be made.

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'Iamque opus exegi, quod nec Iovis ira nec ignis
nec poterit ferrum nec edax abolere vetustas'

Ovid Metamorphosis XV